

'Mars mania' and the right to celebrate

Whether there is life on Mars or not, the public response to last week's findings from meteorite ALH84001 should serve to remind scientists why they do what they do.

THE space science community has just enjoyed one of the finest weeks imaginable, basking in the reflected glory of a result that tentatively suggests that there was once life on Mars — capped by a ringing endorsement of its work delivered by an ebullient US president on the White House lawn. Yet parts of the community respond as though they had just been mugged.

Scientists of many disciplines are sceptical about the evidence presented by McKay *et al.*, no single strand of which is either conclusive or even unique. People from the US National Science Foundation's Antarctic Program — which found the rock — and other scientists related to the project feel usurped by the typically brazen manner in which the National Aeronautics and Space Administration (NASA) has sought to take all the credit for itself. Stephen Jay Gould, writing in Sunday's *New York Times*, points out that the result comes as no surprise to palaeontologists such as himself and asks, in effect, what all the fuss is about.

What is all the fuss about? The paper to be published tomorrow in *Science* presents several separate strands of evidence and argues that, in combination, they "could be fossil remains of past Martian biota". Individually, the different strands do not amount to much (see pages 575–576) but, the authors say, "when they are considered collectively, particularly in view of their spatial association, we conclude that they are evidence for primitive life on early Mars".

At which point, enter Dan Goldin, NASA's rhetorically gifted administrator. Goldin has a treacle-filled yarn for every occasion: on 7 August, the grandest one of all, he told the world's media of a telephone conversation the previous evening with his ailing father in Florida. If nothing else, he said, the pending announcement of this scientific discovery had lifted his father's spirits.

For once, Goldin's story rings perfectly true — and *that* is what all the fuss is about. The entire population is gripped in fascination by the contents of this piece of rock — especially the very young and very old, less absorbed than the rest of us in the daily banalities of earning a living. Their fascination runs through all the generations, from the eight-year-old child who earnestly informed the CNN television channel that the paper cannot be right, because the meteorite came from the wrong part of the Red Planet (how he knew this is unrecorded) to Dan Goldin's father.

Modern science is nothing more nor less than the operating arm of the population's insatiable curiosity. This may seem obvious, but, from some of the ways science has been trying to sell itself of late, one would never guess as much. Bereft of its alleged former mission of fighting the Cold War, it is most often presented as a means of fighting an imaginary economic war between various countries that are demonstrably at peace with one another and whose economies are mutually dependent. On other days, science is pitched (not very convincingly) as a route to cheaper health care, or as the saviour of an overstressed global ecosystem.

With the demise of the corporately funded basic research laboratory and of the Soviet Union, virtually all scientific research is now paid for by the public, through its elected representatives. The public should pay for science because the people want to explore the frontiers of knowledge. If the people ever tire

of such exploration — or if their tedious yearnings are beneath the consideration of scientists — then the funding should cease. But even in this jaded age, last week's events show that the public's curiosity is as vibrant as ever.

So what if there has been no life on Mars, and the expectations raised in every corner of this planet by the McKay *et al.* paper are gradually deflated by further study? Then we will still be left to ponder the response of the people (pursued breathlessly by politicians and the press) to the possibility of progress, and a timely reminder of what science is for. As Margaret Thatcher once said: "Rejoice". □

The resignation tool

The mass resignation of advisers to the board supposed to regulate air pollution in Los Angeles sets a good example.

NINE out of eleven members of the scientific advisory panel to the South Coast Air Quality Management District (AQMD) in California resigned last week (see page 567). They concluded that, with the recent publication of a peculiarly limp air pollution management plan for the Los Angeles area, their advice had been ignored once too often.

Their principled stand may not reverse the AQMD's policies, which are based on the notion that even if the air in Los Angeles is the dirtiest in the United States, further regulation would do unnecessary harm to the local economy. But, by taking a stand, the nine set a fine example for members of other scientific advisory boards. All too often in this type of situation, such panels sit uneasily in silence, lending false credibility to the inept policies of the bodies they advise.

Western politicians have lost the resignation habit: since Lord Carrington resigned as UK foreign secretary in 1982, over mistakes made by his subordinates in the run-up to the Falklands conflict, no British minister has resigned on a matter of principle, and in the United States the concept is almost entirely forgotten. Donna Shalala, the health secretary, for example, is deeply opposed to the welfare bill which President Bill Clinton has recently agreed to sign, but the question of her resignation has not arisen. As politicians have forgotten when to resign, the public, oddly enough, has forgotten to hold them in any kind of esteem.

Scientists should be wary of falling into the same trap. The more eminent are increasingly enmeshed in a web of advisory boards, especially in the United States, and service seems a virtual obligation for some. But the boards can be too close by half to the agencies that they advise.

In southern California, the efforts of local politicians to extract the teeth of the AQMD had gone too far, and it was time for the environmental scientists, doctors and economists on the advisory panel to call it quits. In doing so, they have scored an immediate success in drawing public attention to the AQMD's policies. They also remind all of us that resignation is sometimes the only adequate declaration of independence. □



Meteorite claims revitalize plans for further Mars missions

Washington. The US space agency began looking this week into how it might revamp — and possibly accelerate — its Mars exploration programme, in the light of new evidence of past life on the planet and a presidential call for a summit meeting to chart a course for the nation's future in space.

A study team representing several National Aeronautics and Space Administration (NASA) centres, universities and the Lockheed Martin Corporation, which is building the first Mars Surveyor spacecraft due to be launched in November, planned to meet this week in Washington DC to assess technical and financial options for changing the current line-up of Mars missions. These plans call for launching Mars Surveyors at each of five opportunities — 1996, 1998, 2001, 2003 and 2005 — at a total cost of approximately \$1 billion.

The dramatic claim last week by a team from NASA's Johnson Space Center in Houston, Texas, that they had found fossils in a Martian meteorite (see pages 575–576) set off a wave of excitement around the world, as well as in Congress and the White House. While the agency's administrator, Daniel Goldin, was careful not to imply that NASA would automatically boost its budget for Mars exploration, he said "we may want

to consider some very bold missions where we bore down real deep, and we bring back samples". A sample-return mission is not currently part of the Surveyor plan, and many scientists are sceptical that the agency can afford one within its current science budget (see *Nature* 382, 481; 1996).

Jurgen Rahe, who heads NASA's planetary exploration effort, said the new study is

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Secrets of life? The surface of Mars is attracting more interest from US policy-makers.

meant as contingency planning only, in case the agency should be asked how, when and at what cost it could mount a sample return mission. "We want to be in a position to give a good answer," he said. The study group, to be chaired by Dan McCleese of the Jet Propulsion Laboratory in Pasadena, California, hopes to have a quick analysis ready for next week's meeting of the agency's advisory group on Solar System exploration.

The "summit" announced by President Bill Clinton, to be chaired by Vice-President Al Gore, is in fact a belated response to a call earlier this summer by Senator Barbara Mikulski (Democrat, Maryland) for a meeting of White House and congressional leaders to discuss issues related to long-term

NASA funding. Mikulski, as well as Republicans on the House of Representatives Science Committee, is concerned that six-year budget projections released by the White House early this year do not include enough money for NASA missions already on the books. Gore's staff said last week that they had just begun working on plans for the bipartisan summit, and are not yet sure

NASA whether it will involve the scientific community as well as politicians. It is expected to take place in early December.

An accelerated programme to study Mars — either directly or through further examination of Martian meteorites — would probably involve other agencies besides NASA, including the National Science Foundation (NSF) and perhaps the Department of Defense, which has pioneered advanced miniaturized spacecraft technology.

Neal Lane, the director of NSF, was careful to point out last week that the meteorite containing the purported fossil was collected by NSF researchers in Antarctica, and that the advanced microscopes used to study the samples resulted from US investment in scientific instrumentation. Richard Zare of Stanford University in California, a member of the team that announced the find last week and the new chairman of the National Science Board, which governs the NSF, took the opportunity to argue for more support for Antarctic research, which, he said, is "woefully underfunded".

International Mars missions may also get a boost from last week's announcement. Although the European Space Agency (ESA) by-passed a proposal called Inter-Marsnet in favour of an astrophysics mission this year, space scientists on both sides of the Atlantic are still hopeful that some remnant will resurface in a future joint mission. An international Mars exploration working group, set up in 1994 to coordinate the plans of NASA, ESA, Russia and other European national space agencies, has its next meeting in December.

By that time, the US space summit will have taken place, three Mars missions (including one from Russia) will have been launched, and the world will have had several months to see whether the claim of past life on Mars holds up to scientific scrutiny.

Tony Reichhardt

India loses vaccine manufacturing project

New Delhi. A US\$80 million Indo-French vaccine project launched in 1989 is being wound up before a single drop of vaccine has been produced. India's Department of Biotechnology says that this is the direct result of the French company reneging on a promised technology transfer deal.

In partnership with India, Institut Marieux (IM) of France was to set up on the outskirts of Delhi one of the world's largest plants for manufacturing measles, rabies and injectable polio vaccines. The agreement included a FF69 million (US\$13.8-million) credit from the French government, given direct to IM as payment for the transfer of technology and equipment to India.

But the project ran into trouble when the ownership of IM — renamed Pasteur Marieux Serum Vaccines (PMSV) — shifted to private hands (see *Nature* 360, 3; 1992). The new company considered that the transfer of the proprietary 'vero' cell technology — which allows mass production of vaccine in fermenters rather than using animals — did not make business sense.

"The company was just waiting for a chance to pull out," says a spokesman for the Department of Biotechnology. The opportunity came when the Indian health ministry decided to use oral vaccine instead of the injectable form intended in the original agreement. PMSV's representative in India declines to comment.

K. S. Jayaraman

Forum to advance trials for AIDS therapies

Washington. US drugs companies and federal agencies are to collaborate in a new forum that will try to agree on the design and funding of the clinical trials that are needed to optimize combination therapies for treating AIDS.

AIDS activists and organizations that pay for health care will also be represented on the 40-member Forum for Collaborative HIV Research, which was announced by Vice-President Al Gore on 2 August.

The forum will address increasing concern about how AIDS researchers can keep track of the efficacy of different

therapies as drugs arrive on the market and are used in various combinations. Some new drugs have been approved by the US Food and Drug Administration under an accelerated approval procedure, which means that they reach the market before the completion of normal, three-phase clinical trials.

The idea of a forum on AIDS research issues arose from an AIDS summit at the White House last December and a meeting last February between Gore and the chief executives of drug companies. The details were worked out in meetings organized in

Washington by the Keystone Center. As the promise of new combination therapies emerged, the meetings agreed that the forum should concentrate on the question of the trials.

Assessing the combinations will require new trial designs, which may, for example, have to accommodate the likelihood that patients will change therapy in mid-trial.

The issue of who should pay for such trials is unresolved. Government agencies and drugs companies both hope that the health-care providers will help to finance them. These providers are facing tough choices and huge bills as patients try to move on to combination therapies, which cost up to \$25,000 a year.

Tony Fauci, director of the National Institute of Allergy and Infectious Diseases (NIAID) and a forum member, says that the body will "serve as a venue where people can identify gaps that exist" in planned trials. It will not have the power to agree on the financing of trials, but will serve as a "catalyst for interaction" between different parties.

NIAID has two programmes for conducting clinical trials, the AIDS Clinical Trials Group and the Community Program for Clinical Research on AIDS, which receive \$130 million a year. But these programmes are not going to pay for the large-scale trials now needed.

The drug companies will not pick up the full cost either, because they do not need the trials to obtain approval for their therapies. Linda Distlerath of Merck, another forum member, says it is "unlikely that any entity can do [the trials] on their own".

The trials "are going to look different from trials we've seen in the past", says forum member David Barr of the Gay Men's Health Crisis, a New York-based AIDS advocacy group. "Everyone realizes this is going to be difficult, but we've got to make an attempt."

The trials will have to decide not just the efficacy of different combinations, but also the best time for physicians to switch from one regimen to another, and to withdraw ineffective therapies.

The trials will aim to provide information for physicians on the treatments that they should be prescribing. Just as important, they will inform health-care providers — including the public Medicaid programme, managed-care organizations and private health insurance companies — about which treatments they should be prepared to pay for.

The forum is in the process of finding a headquarters, hiring a small staff and raising funding, which is expected to come from both public and private sectors. It will start its meetings next month.

Colin Macilwain

German groups debate ethics rules

Munich. The German section of the Pugwash Conferences on Science and World Affairs has criticized a report by the research society, the Deutsche Forschungsgemeinschaft (DFG), calling for a relaxation of the strict regulations under which German scientists have to operate, particularly in biotechnology (see *Nature* 380, 469; 1996).

In a hard-hitting statement published earlier this summer, the Vereinigung Deutscher Wissenschaftler (VDW) calls the DFG report "self-righteous and self-pitying". The real threat to research is not over-regulation, argues the VDW, but the increasing tendency to respond to "pressures to answer short-term economic or medical needs".

VDW was founded in 1959 by nuclear physicists opposed to nuclear armament, including Max Born, Otto Hahn, Werner Heisenberg and Max von Laue.

The DFG report, says the VDW, reveals a lack of understanding by research scientists of public concern about aspects of science such as the use of animals in experiments and genetic engineering. Yet "research is paid for by society and therefore is also accountable to society", it argues.

One strong point of disagreement concerns two articles on fundamental rights in the German constitution that appear to conflict when applied to research on human embryos. One article guarantees the freedom of research, the other the right to life and freedom from injury.

The DFG had argued that, when research aims to protect life in the long term, the freedom of research article should take precedence. But the VDW argues that there should be no exceptions to the primacy of the right to life.

As new developments in biotechnology and embryo research impinge directly on this right, says the VDW, public fears are legitimate and should not simply be ignored. It calls for either formal public hearings or referenda on "complex moral problems" such as embryo research before further DFG projects in such

controversial areas are selected for funding.

Such important decisions should not be left to "elitist ethics commissions", says Hans-Peter Bull, professor of law at the University of Hamburg, a co-author of the VDW position paper. The paper also says that researchers should not be exempt from legal liability if applications of their work go wrong.

The DFG has been fighting for years against the bureaucratic obstacles facing biomedical researchers, with some recent successes. So far it has responded cautiously to this attack from within the academic community. At its annual meeting in Leipzig in June, DFG president Wolfgang Frühwald gave a formal welcome to the document, saying that the aim of its report had been to stimulate such discussion.

Along with Hubert Markl, the new president of the Max Planck Society, Frühwald has offered to conduct an open debate on the subject.

But Rüdiger Wolfrum, director of the Max Planck Institute for International Law in Heidelberg, co-author of the DFG report, is sceptical of the VDW's demand for more participation by the lay public in complicated scientific decisions. "The complexity of gene technology does not lend itself to the formulation of questions that can be answered with a simple yes or no," he says.

Wolfrum also rejects the idea of imposing legal liability on basic researchers in the event of undesirable consequences of research applications. Apart from the principle involved, the gap between basic research and the development of products is so wide that it would be impossible to prove guilt.

Quirin Schiermeier

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**Frühwald: welcomed
ethics discussions.**

Fotostudio Querbach

Science advisers resign over smog policy

San Francisco. Nine of the 11 scientific advisers to southern California's smog control agency resigned last week, complaining of increasingly lenient policies that they said put public health at risk. They specifically criticized a new plan to control smog, announced earlier this month, which they said would abandon significant new control measures.

The South Coast Air Quality Management District has been a world leader in the development of regulations and technology to control air pollution. But in recent years the district has cut spending and shifted its regulatory posture to ease the pressure on businesses and motorists to cut emissions.

The specialists who resigned from the agency's advisory committee included chemists, economists, health experts and environmentalists. They said they believed that the agency's policies were no longer directed towards achieving healthy air quality, especially with regard to meeting federal government standards on ozone and fine particles.

In their resignation letter, eight members of the group pointed to signs of "waffling" on policy, including "an absence

of enforcement", "a proposal to dismantle the world's premier air monitoring network" and the "absence of concerted rule-making in the proposed new plan". The ninth adviser resigned in a separate letter that agreed with the others' concerns.

Reactions among the governing board for the district ranged from shock and distress to relief, says Tom Eichhorn, director of communications. One board member called the resignations "a breath of fresh air", Eichhorn says. Senator Ray Haynes (Republican, Riverside) told the Los Angeles Times that he had been sceptical about the value of the panel and concerned about the misuse of science in environmental management.

But Larry Berg is an environmental and energy consultant and expert in political science who served on the board for 11 years. He says he expects the resignations to stimulate the public, environmentalists and academics to become more vocal in opposition to the board's changing posture. "This is a very significant event," he says.

Berg, who left the board in 1993, criticizes the smog control plan as an anti-science, anti-intellectual approach which discarded peer-reviewed studies in deciding

what level of pollution is a threat to public health and how to reduce it.

The plan is based on a new analysis which concludes that Los Angeles, Orange, Riverside and San Bernardino counties will meet federal guidelines for pollution control by 2006 without bringing in controversial measures such as cutting the number of vehicle trips.

The draft plan says: "With the new data and tools, it can now be determined that fewer additional reductions in emissions are needed to meet the federal ambient air quality deadlines." But it acknowledges that southern California still has the worst air quality in the United States.

Those who resigned from the advisory panel, who said they had no part in developing the plan, criticized its predictions as over-optimistic. Most had been on the advisory committee for at least a decade.

The district had pioneered pollution control, reducing emissions by 50 per cent over ten years. Observers say it now appears to be bowing to business and political interests which had blamed its policies for severe job losses.

Sally Lehrman

Japan shuns radishes after 'possible link' to *E. coli*

Tokyo. Japan's health ministry has set off a public scare by announcing that white radish sprouts, a commonly eaten vegetable in Japan, may be one source of the recent mass infection of schoolchildren with a potentially deadly strain of *Escherichia coli*.

The statement has also stirred up controversy because there is as yet no direct evidence linking the radishes to the poisoning outbreak. The premature announcement reflects public pressure on the ministry to trace the source of infection, which has caused sickness in more than 9,000 people, most of them children, and killed nine.

Last week the ministry released an interim report suggesting that white radish sprouts (*kaiware daikon*) produced by a farm in Habikino, near Osaka, may be the source of the 0157 strain of *E. coli* which caused food poisoning in July among more than 6,000 schoolchildren in Sakai city, also near Osaka, and at a nursing home in Habikino (see *Nature* 382, 290; 1996).

But the evidence is only circumstantial. The *E. coli* has not been detected in the radish sprouts or at the farm. The ministry's investigation has simply established that the sprouts were common to meals eaten at schools in Sakai and at the nursing home just before the outbreak, and that the DNA make-up of the bacteria at the two locations is very similar, suggesting a common source of infection.

Despite the lack of firm evidence, supermarket chains have reacted by taking radish sprouts off their shelves, regardless of origin, and restaurants have taken the vegetable off their menus. Radish farmers throughout



Root of the problem: evidence linking *E. coli* 0157 and the radishes is circumstantial.

Japan are protesting that the ministry's action threatens them with financial ruin.

Naoto Kan, the health minister, reportedly made a "political decision" to announce the ministry's suspicions before gaining hard evidence. "We cannot conclude that white radish sprouts were the contagion source, but we cannot rule out the possibility that they were the source of infection," Cabinet members reported him as saying at the time the ministry's report was released.

Kan is no stranger to controversy. Earlier

this year he received wide public praise for exposing his ministry's failure to halt the spread of HIV infection among Japanese haemophiliacs in the 1980s, but he is being severely criticized on this occasion.

As public confidence in sprouts collapsed, Kan stressed that the vegetable was only suspected of being the source. "Since 0157 has not been detected in the radish sprouts and production facilities, we don't have all the objective facts," he admitted at a meeting of the health committee of the lower house of the Japanese parliament.

Scientists have expressed doubts that the radish sprouts are the primary source of infection. The 0157 strain of *E. coli* occurs naturally in the intestines of cows and can be carried in the animals' meat or faeces. Secondary contamination can occur when other food comes into contact with contaminated meat or the bacteria are transmitted by infected humans.

Radish sprouts are grown in urethane sponges under hydroponic cultivation. An inspection of the Habikino farm on 24 July, after the outbreak of food poisoning, failed to detect the bacteria in either the sprouts, seeds or water for the plant. A second, more thorough, inspection has been made, but results are not yet available. Very small numbers of the bacteria — probably as few as ten — can cause infection, so it is difficult to trace their source.

David Swinbanks

US science foundation gets the go-ahead for more centres

Washington. An independent assessment has given the National Science Foundation (NSF) the green light to support a new round of Science and Technology Centres (STCs) based at universities. But the NSF should pay more attention to the choice of directors for the centres, the report says.

The study, by a panel of the Committee on Science, Engineering and Public Policy (COSEPUP) chaired by William Brinkman of Bell Laboratories, found that the 24 existing STCs were doing "world-class research" and that the NSF is "getting a good return" on the \$60 million it spends each year on them.

The centres, which were initiated in 1987 by the administration of President Ronald Reagan, initially drew criticism from university scientists who thought that the money that was available could be spent more wisely through NSF's normal means of providing peer-reviewed grants to individual researchers.

The existing centres, which range in their fields of interest from the Southern California Earthquake Centre at the University of Southern California to the Center for Molecular Biotechnology at the University of Washington, Seattle, were set up as pilots in 1989 and 1991. Their NSF funding is due to end after 11 years.

The COSEPUP verdict makes it likely that the NSF will decide to fund new centres when the old ones expire. David Schindel of the NSF's Office of Science and Technology Infrastructure, which oversees the centres, said that if this happened, people from the established centres would be free to compete to host them, if they have new ideas. "The assumption is that any organisation can produce a proposal — but not just to renew existing centres," he says.

COSEPUP found that, where problems had arisen with the centres, they could be attributed to the administrative capabilities of their directors. It calls on the NSF to "place greater weight" on that person's abilities in any future selection process.

It also found that some centres had placed too much emphasis on education initiatives, especially outreach to high schools, sometimes at the expense of research. "Research and the undergraduate and graduate education linked to it should be paramount" for the centres, the report says.

The NSF will bring proposals for a new competition to its governing board, the National Science Board, in time for its November meeting, Schindel adds. Other advice, including that of a special advisory committee chaired by Carl Lineberger of the University of Colorado, will also be taken into account, he says. Colin Macilwain

Drugs institute gives grant for cocaine vaccine

Boston. The development of a treatment for cocaine addiction is now a "top priority" for the US National Institute on Drug Abuse (NIDA), according to Alan Leshner, its director.

The institute last week announced a \$700,000 grant to ImmuLogic Pharmaceutical Corporation, a biotechnology company based in Waltham, Massachusetts, to complete animal testing of a vaccine to treat cocaine addiction, and to devise plans for human testing of the compound next year.

No medicine is currently available to treat addiction to cocaine, says Leshner, although there are an estimated 2 million heavy users of the drug. Counselling, psychotherapy and

develop a vaccine to treat nicotine addiction.

David Self, a neuropsychopharmacologist at Yale University School of Medicine, believes the strategy used by ImmuLogic, as well as by investigators at the Scripps Research Institute (Carrera *et al. Nature* 378, 727–730; 1995), is a "valid approach".

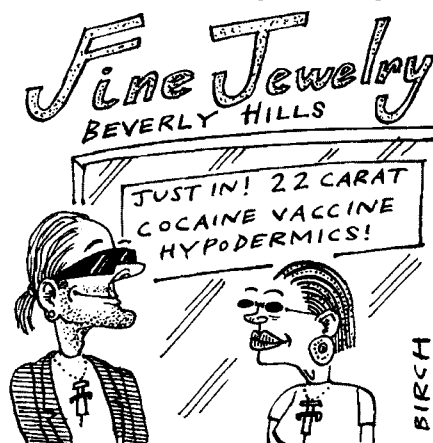
Yet it has a few shortcomings, he says. "It may be possible to overcome the antibody effect by taking more cocaine or by choosing another drug of abuse like methamphetamine or opiates." The vaccine, he adds, would not treat the "core symptomatology" of drug addiction — the intense drug cravings that often lead to relapse. Such cravings are thought to be caused by neurobiological adaptations that occur in the brain in response to chronic drug exposure.

Fox maintains that experiments will be done to find out how readily someone can overcome the vaccine through higher doses, but concedes that people can always switch to another drug. "This approach is not likely to be effective unless patients have some level of motivation," she says. "The good news is that motivation can be encouraged from the outside through counselling."

Another advantage, she points out, is that the antibody approach should be compatible with other experimental drug therapies aimed at blocking dopamine receptors, believed to be the molecular target of cocaine in the brain.

"We'll never have a single approach that works for all people," Leshner says. "Some people will respond better to a drug that targets the underlying mechanism of addiction, while others may do better with a drug specifically aimed at blocking cocaine's effects." In the absence of any available treatments for cocaine today, he says, "we should pursue a variety of strategies, provided the methods are based on sound science".

Steve Nadis



self-help groups are the only options available at the moment. About 400,000 people seek medical assistance or treatment each year, but half of those who have successfully withdrawn from the drug later relapse.

The vaccine to be tested is a cocaine derivative coupled to a carrier protein, which is predicted to elicit an immune response for months after being injected. If a vaccinated person were to take cocaine, antibodies would attach to the cocaine molecules and inhibit their passage into the brain, thereby blocking the psychostimulatory effects of the drug. ImmuLogic's studies in rats show that vaccine-induced antibodies really do limit access of cocaine to the brain. Moreover, rats injected with cocaine-specific antibodies fail to be stimulated by cocaine, and reduce their self-administration of the drug accordingly.

"The medication won't affect drug craving directly, because so long as cocaine is not in the system, these antibodies won't do anything," explains Barbara Fox, an immunologist and vice-president and director of the drug abuse programme at ImmuLogic. "But the treatment will make cocaine less gratifying and could keep a recovering addict from going on a full-scale binge." The company plans to use the same approach to

German staff ruling

Munich. Germany's federal constitutional court last week confirmed the right of universities to employ postgraduate and post-doctoral scientists on short-term contracts. The court has taken more than a decade to resolve the case. It was brought in 1985 by trade unions, which had argued that all academic staff should be on lifelong contracts.

Almost 200,000 young researchers are now employed on short-term contracts at German universities, which, with science associations, reacted to the long-awaited decision with relief. If short-term contracts were inadmissible, they say, a few scientists of a single generation could block all university research positions for 20 years. Q. S.

Australian universities face disruptive changes

Sydney. Australia's education minister, Amanda Vanstone, announced last week that the government is to cut university funds by 5 per cent, despite an agreement to keep budget decisions secret until later this month.

The A\$1.8-billion (US\$1.4-billion) cut will be phased in over four years. To sweeten the pill, Vanstone pledged to fulfil a promise to boost funds for research infrastructure by A\$90 million and for postgraduate awards by A\$40 million over the same period.

The cut is less than had been feared.

Vanstone hinted in May that a cut of up to 12 per cent might be imposed (see *Nature* **381**, 456; 1996). This prompted a prolonged and angry campaign by academics, including strikes by university students and staff. Protests are continuing, however, particularly over increases in fees that double the cost to students taking science degrees.

The cuts are part of an attempt by prime minister John Howard's new Conservative government to reduce public spending to trim a budget deficit of A\$10 billion, without raising taxes. In their election campaign, the

Conservatives had pledged not to cut university operating funds.

Vanstone's optimistic assertion that "universities will be very happy" with the package has been denied by nearly every vice-chancellor. Gavin Brown, head of the University of Sydney, calculates a cut of 15 per cent in real terms until 2000.

Vanstone has signalled a major inquiry into long-term plans for higher education, which the vice-chancellors had sought. Some, like Steven Schwartz of Murdoch University in Perth, Western Australia, are calling for "rational" solutions to the financial changes through cooperation between neighbouring universities.

Funding pressures are expected to force universities to modify their egalitarian claims to funding parity in both teaching and research. Research now looks set to become concentrated in specialized institutions, particularly the 'group of eight' long-established universities, which already command 80 per cent of research funds.

Enrolments for science-based courses are likely to suffer because the current, flat-rate contribution of around A\$2,500 per year (23 per cent of real costs averaged over all courses) is being increased differentially.

While students on arts and social science courses face an increase of 35 per cent, science and engineering students will have to pay nearly twice the current fees. Students of medicine, dentistry, veterinary science and law will pay 125 per cent more.

Vanstone says that the fee increases are fair because of the enhanced earning power of graduates. Graduates will now be obliged to begin grant repayments (through taxation returns) when their salaries reach A\$20,700, compared with the current level of A\$28,500.

Universities will be allowed, for the first time, to charge full fees to some Australian students, an option previously available only for students enrolling from overseas.

Joe Baker, president of the Federation of Scientific and Technological Societies, has not been silenced by his recent appointment to the prime minister's Science and Engineering Council from branding the effect of the cuts on science as giving "all the wrong signals".

Baker questioned "the logic in making science a less attractive career to our best and brightest students" and contradicted Vanstone's claim that graduates have higher incomes. "Too many young research scientists face underpaid, uncertain careers on short-term funding."

Political manoeuvring has begun in an attempt to block the cuts, especially the increased course fees, in the Senate, where the balance of power is held by the opposition Labor Party, the Australian Democrats and three independents.

Peter Pockley

Report advises against privatization

London. A UK government committee set up to investigate options to privatize three public sector research establishments including the British Geological Survey (BGS), is understood to have advised ministers against private ownership.

The committee submitted its report to Ian Lang, president of the Board of Trade, at the end of last month. A separate 'competitiveness' committee of cabinet ministers will consider the review in October. Controversially, the three establishments do not officially know the report's contents.

The report is part of the government's continuing 'prior options review', which is investigating the possibility of privatizing more than 40 public-sector research establishments. It also covers the Centre for Coastal and Marine Sciences in Plymouth and the Centre for Ecology and Hydrology in Oxfordshire, as well as the BGS. All three establishments form part of the Natural Environment Research Council (NERC).

None will comment on the steering committee's recommendations. But all have vigorously lobbied against privatization. The BGS even published in full its submission to the steering committee.

Peter Cook, director of the BGS, says that it is not possible for a private-sector organization to own or manage the BGS without compromising the organization's "excellence, impartiality, relevance and authority". The BGS is already being run on business lines, he says. Profits from a privatized BGS would "not necessarily benefit the science".

But ministers are not bound by the recommendations. The first batch of establishments reviewed — including some linked to the Biotechnology and Biological Sciences Research Council and the Ministry of Agriculture — are now undergoing a second review of future options. Ministers are believed to have disagreed with the recommendations of the original prior-options review committee.

The government's decision to withhold the steering group recommendations from

the three establishments has fuelled a growing controversy about the lack of openness of the prior options review process. The recommendations for the first batch of reviews have also been kept secret.

Cook says that while he understands the constraints under which ministers and civil servants operate, restricting the report to ministers and the steering group "has not been helpful".

But John Krebs, chief executive of NERC, points out the steering committee's report is not the result of "a one-way process". All three research establishments

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REASONS

Work of the British Geological Survey could be compromised by privatization.

were "closely involved in the development of the steering committee's thinking", he says. But "in all these processes, as much openness as possible is desirable".

A spokeswoman for the Department of Trade and Industry, however, rejects suggestions of a policy of secrecy about the recommendations of prior-options review steering committees. "Research councils do know what the recommendations are," she says, "because they sit on the review committees".

Ehsan Masood

Tobacco company found negligent in lung cancer case

Washington. In a landmark decision last week, a jury in Jacksonville, Florida, ordered Brown & Williamson Tobacco Corporation to pay \$750,000 to a 66-year-old man who sued the company after he got lung cancer. Jury members said they were swayed by documents purloined from the company in 1994 which admitted that nicotine is addictive. The jury found the cigarette maker negligent for withholding information about how dangerous its products were.

Stocks in the \$45-billion US tobacco industry fell immediately. Only once before has a jury ruled in favour of a complainant in a similar case; that 1988 New Jersey decision was reversed on appeal.

Brown & Williamson, which called the verdict a "product of error", plans to appeal. The complainant, Grady Carter, a retired air-traffic controller, smoked the Lucky Strikes brand of cigarettes from 1947 until his cancer was diagnosed in 1991. His cancer is in remission.

In interviews with *The Wall Street Journal*, jurors said that documents smuggled out from the company in 1994 were instrumental in their decision. Also important was a videotaped deposition by a former chief executive of Lucky Strikes' former manufacturer, American Tobacco, bought by Brown & Williamson last year. In it, Robert Heimann questioned the validity of all medical research linking smoking to disease. □

Waste goes home

Hong Kong. A large shipment of plastics waste from the United States that has been stuck in Hong Kong for more than a month after Chinese authorities refused to accept it (see *Nature* 382, 484; 1996) will be sent back to the United States. The 200-tonne ship-

ment, including plastic bags from US supermarket chains, was to have been recycled in China. But China is clamping down on such trade and rejected the shipment because it contained rotting food.

The waste will be sent back to the United States in batches over the next few weeks. But environmentalists warn that there is nothing to stop it coming back again. Hong Kong trades in millions of tonnes of such waste each year, most of it destined for China. □

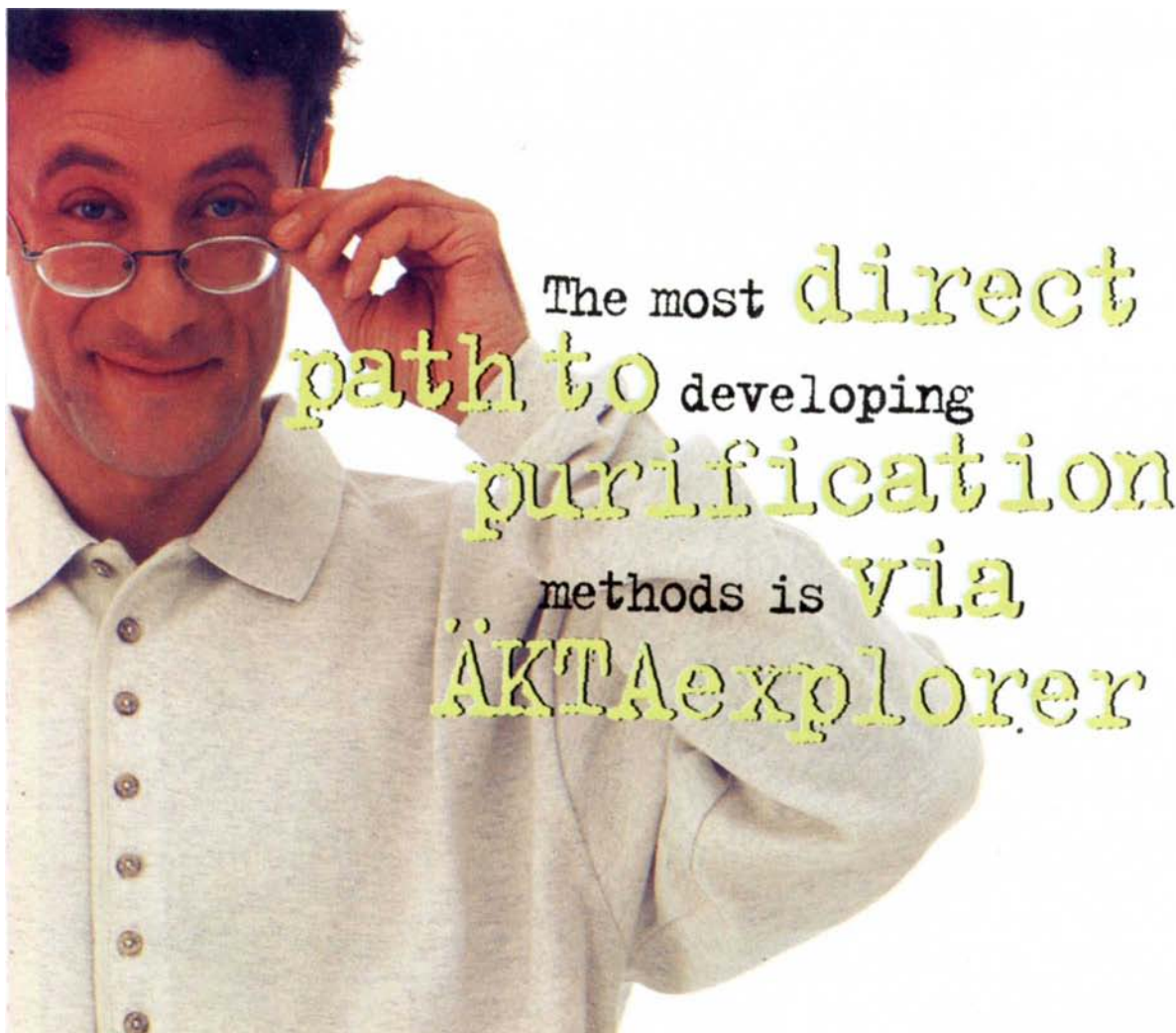
French test sites study

Paris. The Vienna-based International Atomic Energy Agency (IAEA) announced last week that it has completed the first phase of a radiological study of French nuclear test sites in the South Pacific. The eleven scientists from five countries and officials from IAEA who are taking part in the study, collected terrestrial and marine samples from the Mururoa and Fangataufa atolls, where France has carried out 181 tests since 1975. The results will be made known later this year. The second phase of the investigation, a geological study of the area, will be completed by the end of next year. All operations are being overseen by an international advisory committee that includes representatives of the South Pacific Forum, the UN Scientific Committee on the Effects of Atomic Radiation, the World Health Organization and the European Commission. France has paid US\$ 1.8 million towards the costs of the study. □

World space funding

Paris. Space funding has levelled off everywhere, except in Japan, and even there spending increases are slowing down, according to a report, *Government Space Programs, Worldwide Prospects: 1996-2000*, from the Paris-based consultancy Euroconsult.

Space projects remain almost entirely government subsidized. Budget cuts in industrialized countries have hit military space projects the hardest. Once-fashionable microgravity research has been



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one of the main victims of civilian programme cuts. Space agencies are "less disposed towards funding it", says the report, and tend to revert to conventional methods for research such as parabolic flights. Industry sees commercial microgravity research as too expensive.

The report estimates that spending on space this year will be US\$37 billion, three-quarters of it in the United States, which divides its funding fairly equally between the military and civilian sectors. It predicts that US spending will remain flat for the rest of the decade, with a slight shift in its allocation in favour of defence. □

Tropical forests still in decline

London. The annual rate of tropical forest loss is not diminishing, despite the increased awareness of the problems. So concludes a new report by the Consultative Group on International Agricultural Research (CGIAR). Nearly half of the Earth's remaining 2 billion hectares of tropical forest could be lost to agriculture, it claims, and much of the rest to logging.

Ismail Serageldin, chairman of CGIAR and also World Bank Vice-President for Environmentally Sustainable Development, calls for a 'comprehensive effort on a solid scientific basis to attack the root causes of deforestation', which, he says, are caused by 'poverty, rising population, bad natural resource management and distorted environmentally sustainable logging practices'. □

Nuclear accident claims

London. The UK Ministry of Defence has denied claims made in the British press that alleged accidents at a nuclear base during the 1950s and 1960s contributed to elevated concentrations of radiation in the surrounding area.

A spokeswoman for the ministry says there has been "no accident involving nuclear weapons in the UK involving the release of materials that have posed a hazard to the public". She acknowledges that a

number of minor incidents may have taken place in the past. Non-nuclear accidents — or those involving dummy bombs — "cannot be ruled out", she says.

The reports in the weekly *Observer* claim two fires took place at the Greenham Common, Berkshire, air base in 1957 and 1958, the former of which involved a "loaded nuclear bomber". The reports are based on correspondence between scientists investigating high levels of enriched uranium-235 in the vicinity of the air base.

A Royal Air Force operations record book from May 1959 alleges that a 2,000-pound nuclear bomb was "accidentally jettisoned" onto the hard-standing at the air base at Wittering, Lincolnshire, causing "severe damage to the weapon". □

Physicist Mott dies, aged 90

London. Sir Nevill Mott, Cavendish professor of experimental physics at the University of Cambridge and winner of the 1977 Nobel prize for physics, died last week, aged 90. Mott (pictured right), who was Master of Gonville and Caius college, Cambridge from 1959 to 1966, jointly won the prize for his work on the 'theoretical investigations of the electronic structure of magnetic and disordered systems'. A full obituary will be published shortly in *Nature*. □



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Damning verdict on cold fusion

SIR — Del Giudice and Preparata¹ complain about the report and comments in *Nature*^{2,3} that an Italian court had rejected the libel action that Drs M. Fleischmann, S. Pons, T. Bressani, E. Del Giudice and G. Preparata had brought against the editor, publisher and scientific editor (Giovanni Maria Pace) of *La Repubblica* newspaper, claiming 8 billion lire. Pace had written of cold fusion as being similar to scientific fraud.

La Repubblica asked me to provide the scientific and historical evidence⁴, so I should like to mention some facts missing from Del Giudice and Preparata's letter, such as that the court not merely rejected the claim of the five but also awarded costs against them.

Del Giudice and Preparata write that the comments in *Nature*^{2,3} "might convince readers that an Italian court has actually found a proof of misconduct by Martin Fleischmann, Stanley Pons and the three Italian scientists", but in fact *Nature* did not make such accusations — it simply said that the five had lost a libel case and quoted parts of the judgement.

The court judgement was very skilfully written: it does not give a clear scientific judgement on cold fusion but rather says the evidence is such that it is not unreasonable for a journalist to express opinions similar to those of Pace. When the US government wished to make a scientific judgement on cold fusion, it appointed a panel of 22 distinguished scientists who severely criticized the work of Fleischmann and Pons and concluded that there was no compelling evidence for cold fusion, and the court referred to the book⁵ by the co-chairman, John Huizenga.

Another problem that was skilfully solved in writing the court judgement was that the first judge, who later left the case, had appointed a single court-consultant, and both parties were asked to present evidence to him so that he could decide the scientific merits of cold fusion.

It might have been expected that the choice would have been a scientist of international reputation respected by all, with a knowledge of all aspects of the problem (nuclear physics, materials science, electrochemistry and so on) and who could conduct conversations in English without an interpreter. Unfortunately, the person selected had none of these qualifications. Thus, when it was written that "the cells with electrodes of treated palladium with heavy water solutions undoubtedly produce an unexplainable quantity of heat", it does not give an opinion as to whether this was an artefact or had a scientific origin — but its scientific origin could not be fusion without nuclear products such as neutrons or tritium being recorded. Note also that

evidence was submitted that the majority of such experiments did not find excess heat and the best ones rarely found any.

The future prospects of cold fusion were given as a series of escalating claims. The court judgement mentions that in July 1989, Pons gave an interview⁶ where he is photographed with a cell that he claims is a boiler of which he said: "It wouldn't take care of the family's electrical needs, but it certainly could provide them with hot water year-round"; and "Simply put, in its current state it could provide boiling water for a cup of tea". Frequent claims such as these caused the court to write that Fleischmann and Pons had lost touch with reality.

The Sixth International Cold Fusion Conference is to be held from 13 to 18 October near Sapporo in Japan. It is sponsored by the New Energy and Industrial Technology Development Organisation, which is an agency of Japan's Ministry of International Trade and Industry. Those attending will be delighted if Pons can bring his boiler and use it to make us all a cup of tea.

Douglas R. O. Morrison

CH-1296 Coppet,

Switzerland

e-mail: drom@vxcern.cern.ch

1. Del Giudice, E. & Preparata, G. *Nature* **381**, 729 (1996).

2. *Nature* **380**, 369 (1996).

3. *Nature* **380**, 367 (1996).

4. *Nature* **363**, 107 (1993).

5. Huizenga, J. *Cold Fusion — The Scientific Fiasco of the Century* (Univ. Rochester Press, Rochester, NY, 1992).

6. *Desert News*, Salt Lake City, 8 July 1989.

Smoke signals

SIR — I enjoyed John Ashby's *cri de coeur* about the media's handling of human health issues (*Nature* **382**, 109; 1996), especially his recommendation for a better general education in the scientific process. But before he dons his shining armour and charges Giant Ignorance, perhaps he should consider some minor issues.

(1) The scientific process is always easier to describe after the event, whereas scientists apply its principles beforehand. Even within the hallowed scientific laboratory, hypotheses become overly strengthened by the scientist's own convictions, sometimes long before the necessary experimental evidence is available. A good example is the long-held belief of many genetic toxicologists that mutagenicity=carcinogenicity=teratogenicity. A genetic toxicologist well known to both Ashby and me once took a significant proportion of my precious supply of thalidomide in order to follow up the possibility that this teratogen was also a mutagen, in spite of overwhelming evidence in the literature to the contrary. If we cannot learn these lessons ourselves, how

can we expect to teach them to others?

(2) It is a dangerous challenge to the media when a scientific representative of a large chemical industry, with interests in the manufacture of phthalate esters, plays down the potential toxicity of these chemicals.

(3) Finally, I urge Ashby and others not to adopt a diet of three hamburgers a day. It might have a more adverse effect on their health than a normal diet accompanied by passive smoking.

Oliver Flint

Bristol-Myers Squibb,
6000 Thompson Road,
East Syracuse,
New York 13057, USA

SIR — Ashby uses the tobacco industry's campaign on passive smoking as an example of an attempt at educating the public. But this example ironically reinforces the rest of his argument on the ease of creating confusion by the misleading use of scientifically valid statistics. By focusing on the small relative risk of dying from cancer as a result of passive smoking, the tobacco industry conveniently ignores the much greater statistical likelihood, particularly in children, of illnesses such as respiratory infections, asthma and glue ear for those subjected to tobacco smoke. The industry also chooses to ignore the fact that for children, and for adults in many public or workplace environments, passive smoke exposure is not something that they can choose to avoid.

Taken away from the social context in which children cannot escape the tobacco smoke of their families, this 'choose your poison' approach between three hamburgers a day and breathing in other people's smoke is at best meaningless and, worse, offers an insidiously false reassurance, relying on the equally false public perception of eating hamburgers as a comfortable, familiar, harmless act. I hope that attempts by the tobacco industry to 'educate' us will in future fall on sceptical ears.

J. Britton

(Chairman, Tobacco Advisory Group)
Royal College of Physicians,
11 St Andrew's Place,
Regent's Park,
London NW1 4LE, UK

Better late...?

SIR — If Chadwick discovered the neutron in 1904, as *again* stated in your self-advertisement in the issue of 25 July 1996, why did *Nature* wait for close to 28 years (129, 312; 1932) before reporting it?

Maurice Guéron

Ecole Polytechnique,
91128 Palaiseau, France

■ *Touché*. — Editor, *Nature*

Support Irish science

SIR — Researchers in Ireland continue to compete internationally as best they can, but we still lack any coherent long-term policy for the development of basic research. Our industries fail to appreciate the need for highly trained, competent researchers beyond the PhD qualification. We have failed miserably to inculcate a culture of enterprise and innovation.

In 1994, the Science, Technology and Innovation Advisory Council (STIAC) was set up by the then Minister for Commerce and Technology, Mr Seamus Brennan, to advise government on how best to improve the effectiveness of the national science and technology system in contributing to national economic development. The resulting Tierney Report made 160 individual recommendations that underpinned a series of detailed and wide-ranging measures aimed at generating the dynamism necessary to create a highly competitive economy and to make a significant impact on the problem of unemployment.

The recommendations dealt with issues ranging from increased expenditure on research and development (R&D) by business to integrated tax measures to stimulate commercial R&D, intercompany cooperation programmes, earmarking of funds for new R&D in both multinational and indigenous companies, funding for R&D in the food industry, venture-capital funding, technology acquisition and support for patents.

Increased incremental costing over existing base-level expenditure was estimated at £42 million for the first year, rising to a plateau of £82 million after five years. While STIAC recognized that the reallocation of public resources of this magnitude would take time, it recommended an immediate injection of £25 million to fill the most urgent gaps.

During the second half of 1995, the Travers Task Force examined the STIAC report with a view to advising government on the immediate implementation of the recommendations. The results of this exercise, which had no formal input from the research community, are subject to the Official Secrets Act and have never been published. On 19 March 1996, the current Minister for Commerce, Science and Technology, Mr Pat Rabbitte, announced the allocation of an additional £4 million for science and technology in this year's budget to begin implementing the recommendations of the Tierney Report. Any increase in discretionary funding to science and technology is welcome, but the additional funding represents only 10% of the cost of implementing the first year of the programme.

There is an inherent technological and innovative weakness in small and medium-sized enterprises (SMEs) in Ireland. In

terms of R&D spending, the indigenous sector performs about half as well as its multinational counterparts. About 75% of the manufacturing companies in Ireland — almost by definition indigenous SMEs — have only a minimal technological capability. A wonderful opportunity has been missed to launch innovation once and for all and to enable technically qualified young graduates and technicians to begin to inculcate a spirit of innovation within our indigenous SMEs. In the process, many new high-technology skilled jobs might have been created. But the opportunity has been lost because the government, despite having set up an expert group to advise on what needs to be done, has once again failed to make the hard financial decision to implement the programme outlined by the STIAC group. We have instead an *à la carte* selection of some of the STIAC recommendations, with the £4 million spread thinly over a dozen different initiatives, including technology services and techstart schemes, basic research and hopefully the promotion of the awareness of science, technology and innovation.

In recent years, successive Irish governments have poured large sums of money on an *ad hoc* basis into arts festivals, the Gaelic Athletic Association, Dublin Zoo, Telefis na Gaeilge and so on. Projects and programmes appear to be funded on the basis of popularity or on the whims of topical or sectoral appeal.

There does not appear to be any collective political will in the Irish government to address the innovation question. If Ireland is serious about meeting the commercial and industrial challenges of the next century, now is the time to begin implementing the Tierney Report. It is time for visionary collective cabinet responsibility, with decisions on science and technology policy being made by elected government representatives and not by anonymous mandarins within the Department of Finance.

D. J. Fegan

(Chairman, Irish Research
Scientists Association)
Physics Department,
University College Dublin,
Dublin 4,
Ireland

Peace dividend?

SIR — In the former Soviet Union, students entering the traditional annual Chemistry Contest were usually given tasks that were slightly more difficult than the normal high-school courses. In contrast, this year and last in the Soros Contest, the tasks were based on facts known to most students from their school courses, but which required some additional logical thinking and unusual approaches. There

was also a postal round before the main round to help participants to assess the difficulty of the tasks and their own preparedness. When formulating the postal-round tasks, facts that might not be generally known were specially explained.

The Soros Contest attracted high-school students from all regions of Russia. About 2,000 students in each of three age groups took part. The object of the contest is to find talented boys and girls irrespective of their mastery of the school curriculum in chemistry. In both the postal round and the main round, some tasks were formulated similarly for neighbouring age groups (9th and 10th grades, or 10th and 11th grades). In most cases, such tasks were solved significantly better (in some cases twofold) by younger students.

This phenomenon could be interpreted to mean that the number of gifted boys and girls is increasing every year. There must be a reason for such a rise. No significant changes have recently been made in the teaching of chemistry: old textbooks are usually used and there have been even fewer of them.

The explanation seems to be that Russia in recent years has become more 'friendly' for talented people. Boys and girls in 11th grade were born in 1980, those in 9th grade in 1982; their childhood coincided with Gorbachev's *perestroika*. This is the first generation of children who know nothing of the communist 'pioneer' organization in junior high school, when ideology was controlled by the Communist Party Central Committee. Being able from early childhood to hear and discuss alternative points of view, to suggest hypotheses and try to prove them, to think things over and comprehend them instead of just accepting the only 'true and nonrevisable' theory are probably the advantages that students now in 10th and 11th grades have over the older generations in Russia.

The phenomenon of the development of science in a totalitarian state still needs to be investigated. The sudden fall of communism may have given a spur to the rise in the number of gifted people. If so, we may expect an increase in competition for the right to study at leading technical colleges, and a consequent improvement in students' performance.

There are many young graduates from Russian universities and institutes now working successfully in science and technology in the West. The new generation may be even more productive. Taking into consideration the size of Russia and the potential of leading Russian universities, the influx of new Russian scientists into world science may have a global impact on the development of civilization.

Andrey Nedospasov

Institute of Molecular Genetics,
123182 Moscow,
Russia
e-mail: img@glas.apc.org

BSE and risk to humans

SIR — The developments in the handling of bovine spongiform encephalopathy (BSE) by the British authorities and those of the European Union do not provide much hope for clarifying the human risk issue in the near future. The finding of each new case of Creutzfeldt-Jakob disease and even of suspected cases in either the United Kingdom or any other country in Europe will add to the anxiety of the general public, whether it is rational or not.

Even the immediate withdrawal of all meat of potentially infected cows from the food chain will not convince consumers that beef is safe from that moment on, because past experience has shown that such measures cannot be properly enforced. The present dismal situation of both the beef industry and the consumers is due to previous failure by the UK government to commission adequate research programmes aimed at answering the crucial question of transmissibility of BSE to humans by all possible routes.

The recent report by Lasmézas *et al.*¹ of BSE transmission to macaques has shown that at least 3 years ago scientists were aware of the necessity of such an approach. If at that time adequate experiments had been initiated, we would by now, and possibly even earlier, have had all the information needed. Instead of a study with 3 macaques and intracerebral injection of cow brain only, larger groups of chimpanzees should have been treated with intracerebral injections of BSE brain and beef, and administration of infected cow brain and meat and perhaps even milk by the cutaneous, intravenous and of course the oral route, the latter on a continuous feeding basis.

In the case of BSE, chimpanzees are the experimental animal of choice, not only because of the general argument that they are closer to man than macaques, but specifically because another transmissible prion disease in humans, kuru, is known to be transmitted to chimpanzees not only by infected human brain, but also by other tissues. Oral administration of kuru-infected human tissues, however, failed to induce the disease in chimpanzees^{2,3}. Such a programme, though relatively expensive, would have cost a fraction of the present economic losses.

The argument we have heard, that chimpanzees were not available for this research in the United Kingdom, is obviously invalid, as the Dutch Biomedical Primate Research Centre has a large colony of chimpanzees available for research,

and research institutes in France can dispose of chimpanzees in the Gambia. These sources of chimpanzees and others became well known in the course of the European AIDS research programmes sponsored by the European Union.

D. W. van Bakkum
Comprehensive Cancer Centre
Rotterdam,
PO Box 289,
3000 AG — Rotterdam,
The Netherlands

P. J. Heidt
Biomedical Primate Research Centre,
PO Box 3306,
2280 GH — Rijswijk,
The Netherlands

Little to celebrate

SIR — Celebrating its fiftieth anniversary on 1 August, the Institute of Advanced Studies (IAS) at the Australian National University in Canberra was delighted this year by a review that concluded, among other things, that no other institution in Australia, and few in the world, had achieved as much in research as IAS.

But Peter Pockley's article (*Nature* 382, 3; 1996) makes it clear that there has been little to celebrate. It concluded with a comment from Max Brennan, the chair of the Australian Research Council (ARC), that concern about his advice to government on this review amounted to nothing more than paranoia. Brennan now knows better, through the outrage expressed from all quarters of the globe at his wish to control Australia's leading independent research institution.

Having accepted a procedure jointly agreed with the Australian National University, Brennan invited 70 leading researchers to evaluate many hundreds of submissions and assessments. With three colleagues, Brennan then wrote more than 30 pages of 'advice' which, although by no means unanimously accepted by the council, was tabled in parliament as "the ARC Review of IAS". Making a mockery of the jointly agreed review, and overlooking the substantive evidence, Brennan advised government to give to ARC resources recommended for IAS, and, incredibly, to give ARC responsibility for IAS's future strategic planning and funding levels.

What does Brennan have in mind? Evidently embarrassed by high achievers, and unable to recognize his conflict of interest, he seems determined that, perhaps for the next 50 years, the IAS should be encumbered with the bureaucracy that constrains research in the rest of the country's universities. Australian researchers have to act now if they are to avoid

being further fouled by the barnacles of bureaucracy that will drag them away from the leading edge.

Perhaps the treatment of the IAS review was the exposé that ARC had to have. Under a new government there may be an opportunity to restructure ARC into a credible body in support of research, one that respects peer opinion. It must be kept out of policy, away from the pretexts of strategic planning and from destructive meddling beyond this brief. If integrity and confidence can be restored in the ARC, then the anguish underlying the fiftieth anniversary of IAS can be transformed into a gift to Australia's researchers for the future.

Barry Osmond
(Director)
Research School of Biological Sciences,
Institute of Advanced Studies,
Australian National University,
Box 475,
Canberra, ACT 2601, Australia

Present address: Duke University Marine Laboratory, Beaufort, North Carolina 28516, USA.

Tip of the iceberg

SIR — Daedalus's idea of halting sea-level rise if there is global warming by slowing the melting rate of land ice is only a small part of the answer¹. In future projections of sea-level rise^{2,3}, the major contributor is thermal expansion of the oceans. The melting of alpine glaciers and small ice caps (GSICs) accounts for only about 35 per cent of the total sea-level rise to 2100 under the central projection (some 17 cm out of a total of 49 cm compared with 25 cm for expansion).

Beyond 2100, the relative contribution from GSICs is smaller because their total ice mass is equivalent only to about 30 cm of sea-level rise. Slowing the rate of heat sequestration by the oceans is not the answer, as this will only accelerate the warming of the atmosphere (and add to the ice-melt contribution!). Daedalus may be interested in reading about some of the imaginative geo-engineering 'fixes' that have been suggested by others (such as those listed by the US National Academy of Sciences⁴) before speculating further.

Tom M. L. Wigley
National Center for
Atmospheric Research,
PO Box 3000,
Boulder,
Colorado 80307-3000, USA
e-mail: wigley@ncar.ucar.edu

1. Jones, D. *Nature* 381, 196 (1996).
2. Raper, S. C. B., Wigley, T. M. L. & Warrick, R. A. in *Sea-Level Rise and Coastal Subsidence: Causes, Consequences and Strategies* (eds Milliman, J. D. & Haq, B. U.) 11–45 (Kluwer, Dordrecht, 1996).
3. Warrick, R. A. *et al.* in *Climate Change 1995: The Science of Climate Change* (eds Houghton, J. T. *et al.*) 358–405 (Cambridge Univ. Press, New York, 1996).
4. National Academy of Sciences *Policy Implications of Greenhouse Warming* (National Academy Press, Washington, DC, 1991).

1. Lasmézas, C. I. *et al.* *Nature* 381, 743–744 (1996).

2. Gibbs, C. J. & Gajdusek, D. C. *Res. Publ. Assoc. Res. Nerv. Ment. Dis.* 49, 383–410 (1971).

3. Gajdusek, D. C. & Gibbs, C. J. *Nature* 240, 351 (1972).

Opening a martian can of worms?

Monica Grady, Ian Wright and Colin Pillinger

Mars might once have supported life. The evidence, announced at a NASA press conference last week, is not conclusive, and perhaps not even compelling. But it will probably hasten research that should settle the matter.

In the follow-up paper to be published in *Science* tomorrow¹, McKay *et al.* quite rightly express extreme caution in interpreting their findings. Having eliminated the impossible, as the saying goes, the authors are left with the improbable conclusion that the structures they see under the electron microscope are indeed the remnants of bacteria-like organisms from Mars (Fig. 1). Their argument is that a set of related observations made on the meteorite ALH84001 come together to form evidence for primitive life on early Mars. But each supporting thread can be regarded as weak. Even the premise that the meteorite comes from Mars is disputable. But few scientists involved with the subject doubt that the class of meteorites to which ALH84001 belongs, and thus ALH84001 itself, is martian.

Fractures and gaps within the meteorite contain large patches of carbonate deposits (McKay *et al.* call them 'globules') with a carbon isotope composition that indicates a martian origin². How they formed, however, is still under debate. In particular, there are conflicting values for the formation temperature: major-element chemistry^{3,4} implies a temperature of more than 500 °C, whereas oxygen isotope composition² gives less than 100 °C. Until this difference in interpretation is resolved, a low-temperature hydrothermal (and thus potentially biological) origin for the carbonates cannot be stated with certainty.

Neither do we know *when* the carbonates formed. Faults cutting across them, and their carbon isotope composition, show that they are older than the impact that dislodged this rock, so they were certainly produced on Mars; but a recent report⁵ indicates that they formed as late as 1.4 billion years ago, long after the date adopted by McKay *et al.* (3.56 billion years ago). As well as implications for the age of life on Mars, the age of the carbonate has a direct bearing on the evolution of the

planet's atmosphere and the presence of liquid water on its surface.

The carbonates occur in areas that are rich in organic molecules, specifically polycyclic aromatic hydrocarbons (PAHs), at a concentration of about 1 p.p.m. PAHs are not directly synthesized in any terrestrial biological system but are produced by metamorphism. Their identification in the unmetamorphosed ALH84001 cannot be taken

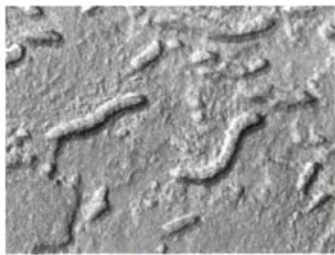
unusually high for a martian meteorite, we stopped well short of claiming the existence of biogenic material. We have also reported a high concentration of organics⁷ in ALH84001 (~ 250 p.p.m., with relative carbon isotope ratio $\delta^{13}\text{C}$ of about -22‰), so one piece of the corroborating evidence requested by Dan Goldin, the head of NASA, is already in place.

In both meteorites, the carbon isotope composition of the material isn't sufficiently different from terrestrial organic compounds to provide an *unambiguous* signature for the planet of origin of the molecules. That might imply that the organics are terrestrial contaminants, but similarly high concentrations are not observed in asteroidal basalts collected from the same areas or in other martian meteorite samples with less stringent contamination control.

If the carbon cycle on Mars is analogous to that on Earth, then there is a fractionation of more than 60‰ between the carbon source (an atmosphere with $\delta^{13}\text{C} \approx +40‰$) and the organics themselves. Here is a plus point: a carbon isotope fractionation of this magnitude generally, but not exclusively, requires a biological mechanism. However, that assumes that the fractionation pathways are similar to those on Earth, where large isotopic effects are produced anaerobically by methanogenic bacteria (photosynthesis produces a maximum fractionation of 20‰). It also assumes that there has been little change in the $\delta^{13}\text{C}$ of the martian atmosphere over the past 1.4 billion years.

Several reasons for caution on the microfossils came from William Schopf, at the NASA press conference: they are a hundred times smaller than any such fossils found on Earth, there is no measurement of the composition of the

cells to show whether they are organic or not, and there is no sign of any cavities within the cells in which fluids necessary for life could reside. Some of the criteria used to identify microfossils in terrestrial rocks are present in ALH84001, but one is not: the carbonates that harbour the



Mars, with close-ups. The orange carbonate 'globules' (bottom right), found in cracks in the martian meteorite ALH84001, may have been formed by hydrothermal activity. The areas around these deposits are rich in organic molecules, but are the 'textures' that appear on their surfaces (such as the elongated 0.7- to 0.8-micrometre features, top left) fossilized bacteria?



as a good biomarker.

We published the first paper documenting organic molecules in a martian meteorite⁶ in 1989, looking at a different Antarctic meteorite, EETA79001. Although we described a concentration of complex organic compounds that is

'microfossils' are found in igneous rock, rather than a sediment.

The magnetite evidence cited by McKay *et al.* is ambiguous even though magnetite — an oxide of iron — has long been recognized as an important marker of biological mineralization. Unfortunately, it can also result from inorganic precipitation. The presence of magnetite in association with iron sulphides in a mineral assemblage sets limits on the partial pressure of oxygen present when the minerals were formed — on Earth such an assemblage reflects anaerobic deposition conditions, that is, a reducing environment.

The individual magnetite grains are tiny — smaller than those associated with most terrestrial microfossils. Furthermore, one of the most basic criteria applied when identifying microfossils is the presence of organized elements, such as a cell wall. The structures shown by McKay *et al.*, as Schopf pointed out, do not appear to demonstrate any such features.

The leap from molecules to microfossils is an enormous one in terms of biological evolution, and there is probably not enough evidence yet for that leap's having been made on Mars. But the interest generated by this paper is so great that we may be able to find out sooner rather than later. There has even been a Presidential statement pledging support for planetary science into the next millennium, so the promotion of research to provide evidence for or against McKay and colleagues' hypothesis may be largely the product of the President's faith.

The onus is now on the scientific community to reproduce the observations and to make a more detailed study of the organic and stable-isotope geochemistry of martian meteorites. As for the more direct approach, NASA has several missions to Mars in the planning stages, and an 'uncommitted' sample-return mission is slated for 2005.

Given the surge of interest in the subject, there will be no shortage of scientists to carry out all this work — and, one hopes, enough money to allow the work to be done. □

Monica Grady, Ian Wright and Colin Pillinger are at the Planetary Sciences Research Institute, The Open University, Walton Hall, Milton Keynes MK7 6AA, UK, and Monica Grady is in the Department of Mineralogy, The Natural History Museum, Cromwell Road, London SW7 5BD, UK.

Life beyond Mars

Christopher F. Chyba

In evaluating evidence for fossil martian life¹ in meteorite ALH84001, as discussed in the preceding article, we should consider the history of similar claims and be cautious. Many hopes for microfossils and biogenic organic compounds in meteorites and ancient terrestrial rocks have been dashed by later work^{2,3}. If the carbonates in ALH84001 were formed at high temperatures in an impact event⁴, a biological interpretation would fail — and this is but one objection.

Few subjects encourage speculation more than the search for extraterrestrial life, and few demand more caution when claiming success. In 1971, the United

States and the mean lifetime of technical civilizations. The challenge of estimating the latter factors on the basis of current knowledge has led to bitter attacks on this entire approach as "totally non-scientific and even meaningless"⁶. But the utility of the Drake equation is shown by the fact that conferences on life in the Universe seem naturally to organize themselves, explicitly or implicitly, according to its terms. The equation is a shorthand for what we are trying to understand, rather than a tool for precise calculation.

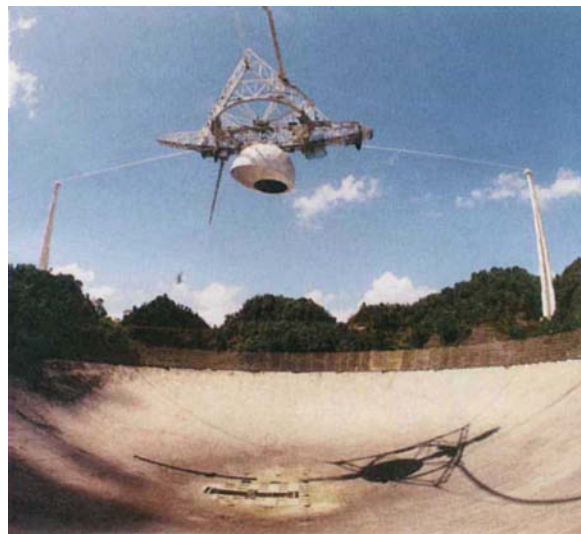
At the time of the Byurakan meeting, it had only just been demonstrated that some meteorites contain extraterrestrial amino

acids and other (non-biogenic) organic molecules relevant to the origins of life⁷. We now know that our own Solar System, as well as interstellar space, is rich with organics. As the Nobel laureate Christian de Duve told the astronomers in Capri, "You have shown that organic chemistry is the most banal chemistry in the Universe." The role that extraterrestrial organics may have played in the origins of life is now an accepted line of research. Even questions as central as the chirality (handedness) of terrestrial biomolecules might be influenced or answered by studies of extraterrestrial organics, were, for example, some meteoritic amino acids to be convincingly shown to favour

left-over right-handedness.

Another advance since Byurakan has been the recognition of the role impacts have played in the history of life in our Solar System⁸. On Mars, impacts may have stripped off the early atmosphere, turning the planet into a freeze-dried desert and rendering any early biosphere extinct — or driving it deep underground. On Earth, giant impacts almost certainly introduced a random extraterrestrial component to the history of life. In general, a biosphere that does not produce a technical civilization (capable of assessing and responding to its particular impact environment) on a timescale shorter than that between giant impacts will be catastrophically disrupted.

The most important advances reported in Capri, however, were discoveries of extrasolar planets. For decades, astronomers have dreamed of obtaining statistics for planetary systems, as they have for stars. But at the time of the Byurakan conference,



Another sign of life: the Arecibo planetary radar, used for mapping nearby objects in the Solar System. An alien radar as powerful as this (30,000 gigawatts) could now be detected more than 150 light years away.

States and Soviet Academies of Science sponsored a conference on "Communication with Extraterrestrial Intelligence" at the Byurakan observatory in Armenia⁵. In the 25 years since Byurakan, some of the meeting's provocative speculation has moved into the mainstream, as was evident last month in Capri at the fifth series of "bioastronomy" conferences aimed at synthesizing ideas from biology and astronomy*.

One way to investigate extraterrestrial life is to inquire into the factors relevant to its origin and survival. Formally, this leads one to the Drake equation⁵, which relates the number of existing technical civilizations in the Galaxy to factors ranging from the rate of star formation and the fraction of stars with planetary systems to the likelihood of the evolution of intelli-

1. McKay, D. S. *et al.* *Science* **273**, 924–930 (1996).
2. Romanek, C. S. *et al.* *Nature* **372**, 655–657 (1994).
3. Mittlefehldt, D. W. *Meteoritics* **29**, 214–221 (1994).
4. Harvey, R. P. & McSweeney, H. Y. Jr *Nature* **382**, 49–51 (1996).
5. Wadhwa, M. & Lugmair, G. *Meteoritics and Planetary Science* **31**, A145 (1996).
6. Wright, I. P., Grady, M. M. & Pillinger, C. T. *Nature* **340**, 220–222 (1989).
7. Grady, M. M. *et al.* *Meteoritics* **29**, 469 (1994).

*Astronomical and Biochemical Origins and the Search for Life in the Universe, Capri, Italy, 1–5 July 1996. IAU Colloquium No. 161.

George Gaylord Simpson could write that "it is still true that not even one Earthlike planet outside our Solar System has been observed objectively or is known to exist in fact. Exobiology is still a 'science' without any data, therefore no science."⁹

Now, suddenly, new detections of planets are being reported frequently, although present searches cannot discover planets much below the mass of Jupiter. It was clear from the presentations by M. Mayor and D. Queloz (Geneva Obs.) and by G. W. Marcy and R. P. Butler (San Francisco State Univ. and Univ. California at Berkeley) that many more will soon be forthcoming. Indeed, another planet, around ν Andromedae, was first announced at Capri (Marcy and Butler), as were several new brown dwarfs (Mayor and Queloz). The best way to keep up to date may now be via the World Wide Web; the Extrasolar Planets Encyclopedia maintained by J. Schneider of the Observatoire de Paris (<http://www.obspm.fr/planets>) lists planets and brown dwarfs, as well as unconfirmed and unpublished reports.

So far, we know of eight massive planets orbiting one M-, two F- and four G-class stars. The searches provide a value for $M\sin(i)$, where M is the planet's mass and i is its inclination to our line of sight, usually unknown. The planets discovered so far have values of $M\sin(i)$ ranging from 0.47 to 6.6 times the mass of Jupiter (M_J). Whether some could in fact be brown dwarfs remains controversial.

Four are 'hot Jupiters', objects within about 0.1 AU of their star (1 AU is the Earth-Sun distance). The existence of such worlds is jarring to those raised to think of our own Solar System, with its giant planets all beyond 5 AU, as 'normal'. The origin of hot Jupiters remains a puzzle¹⁰, but it is now evident that they are common.

One of these hot Jupiters is accompanied by a second planet orbiting at 5–10 AU (Marcy and Butler). Moreover, this same system (55 ρ Cancri) is a binary one in which the G-class star has an M-class companion. The long-standing question over whether binary systems would prove to

have planets has therefore been laid to rest.

These results show that solar systems very different from our own are common, but they do not imply that our own configuration is rare. Selection effects are severe — planets of terrestrial mass cannot be detected, and only planets whose orbital periods are not much longer than the duration of the survey can be reliably identified. The Marcy-Butler survey of 120 stars is nine years old (Jupiter's orbital period is almost 12 years) and they should have detected any planet of more than $2M_J$ orbiting within about 5 AU of its sun.

Where there are planets, there may be life, and where there is life, there may be intelligence. Or maybe not. The further we proceed through the Drake equation, the less our speculations are constrained. In Capri, although there was an excellent presentation on the evolution of cetacean intelligence (L. Marino, Emory Univ.) and talks with such provocative titles as "Why extraterrestrials will be intelligible" (M. Minsky, MIT), some questions were never engaged. Would deciphering a detected signal prove enormously difficult¹¹? Does the fact that humans have had so little success in decoding dolphin communication bear on this question? Will it prove easier to understand technological extraterrestrials than to communicate with non-technical marine mammals?

RNA PROCESSING

Death by decapitation for mRNA

Mick F. Tuite

MESSANGER RNA is a short-lived intermediary in the transfer of genetic information from DNA to protein. Once an mRNA molecule is synthesized and fully processed, it acts as a template for the ribosome to synthesize the encoded polypeptide chain. The cellular abundance of a given mRNA species is a critical determinant of the cellular abundance of the encoded polypeptide chain. Yet, for such a critical component of the gene expression pathway, mRNA is a remarkably unstable molecule, being destroyed by cellular enzymes (nucleases) within a matter of hours or even minutes of its synthesis.

The intrinsic chemical stability of a given mRNA has a strong influence on the level of expression that can be achieved by the gene encoding it. The mysterious pathways that lead an mRNA to destruction are just starting to be unravelled in the yeast *Saccharomyces cerevisiae*, a simple eukaryote. And now our understanding of these processes has reached a major milestone with the identification on page 642 of this issue by Beelman *et al.* of a key enzyme that

A different approach is to bypass Drake-equation controversies altogether, and instead listen directly for signals from alien civilizations. Several such searches are going on, now almost entirely privately funded. The most encouraging results reported in Capri were those of Project Phoenix's four-month search from Australia (J. Tarter, SETI Inst.). They looked for extraterrestrial signals as strong as those produced by powerful radars on Earth coming from any of 202 Sun-like stars, out to a radius of 155 light years. Importantly, Phoenix left behind no mysterious or unexplained signals. It may sound paradoxical, but this is a great success: searches have now reached a level of technical maturity, despite growing anthropogenic interference, where all interference can be recognized and excluded.

The excitement over ALH84001 shows how intense media interest can put pressure on scientific debate. Twenty-five years after Byurakan, as our radio searches mature, it may be time to start thinking carefully about how our society would react to an announcement that an extraterrestrial signal had been detected¹². □

Christopher F. Chyba is in the Department of Geosciences and the Center for Energy and Environmental Studies, Princeton University, Princeton, New Jersey 08544-1003, USA.

1. McKay, D. S. *Science* **273**, 924–930 (1996).
2. Urey, H. C. *Science* **151**, 157–166 (1966).
3. Strother, P. in *Environmental Evolution* (eds Margulis, L. & Olendzenski, L.) 87–101 (MIT Press, Cambridge, MA, 1992).
4. Harvey, R. P. & McSweeney, H. Y. *Nature* **382**, 49–51 (1996).
5. Sagan, C. (ed.) *Communication with Extraterrestrial Intelligence* (MIT Press, Cambridge, MA, 1973).
6. Adler, A. *Atlantic Monthly* **234**, 109 (1974). Reprinted in *The Quest for Extraterrestrial Intelligence* (ed. Goldsmith, D.) 224–227 (University Science Books, Mill Valley, CA, 1980).
7. Kvernolden, K. *et al.* *Nature* **228**, 923–926 (1970).
8. Thomas, P. J., Chyba, C. F. & McKay, C. P. *Comets and the Origin and Evolution of Life* (Springer, New York, 1996).
9. Simpson, G. G. in *Communication with Extraterrestrial Intelligence* (ed. Sagan, C.) 362–364 (MIT Press, Cambridge, MA, 1973).
10. Burrows, A. & Lunine, J. *Nature* **378**, 333 (1995).
11. Morrison, P., Billingham, J. & Wolfe, J. (eds) *The Search for Extraterrestrial Intelligence* (NASA SP-419, 1977).
12. Tarter, J. C. & Michaud, M. A. (eds) *Acta Astronaut.* **21**(2), 69–154 (1990).

mediates a critical step in most, if not all, of the mRNA degradative pathways in this organism.

That mRNA molecules are unstable intermediates in the gene expression pathway was first recognized over thirty years ago^{2,3}, but what was not realized at that time was that every mRNA species has an intrinsic stability that can be quantified by experimentally determining its chemical half-life. In bacterial species such as *Escherichia coli*, a typical mRNA will have a half-life of about two minutes; in yeast it is 10–20 minutes; and in mammalian cells it averages out at several hours. Yet even within a given organism, different mRNA species can have profoundly different half-lives: for example, in yeast the mRNA encoded by the *PPR1* gene has a half-life of one minute, whereas the *PGK1* gene-encoded mRNA has a half-life of 35 minutes⁴. Such differences in mRNA half-life may reflect the differing response times required for the regulation of genes — for example, a rapid regulatory response will be facilitated by unstable mRNAs.

What sets the half-life of a given

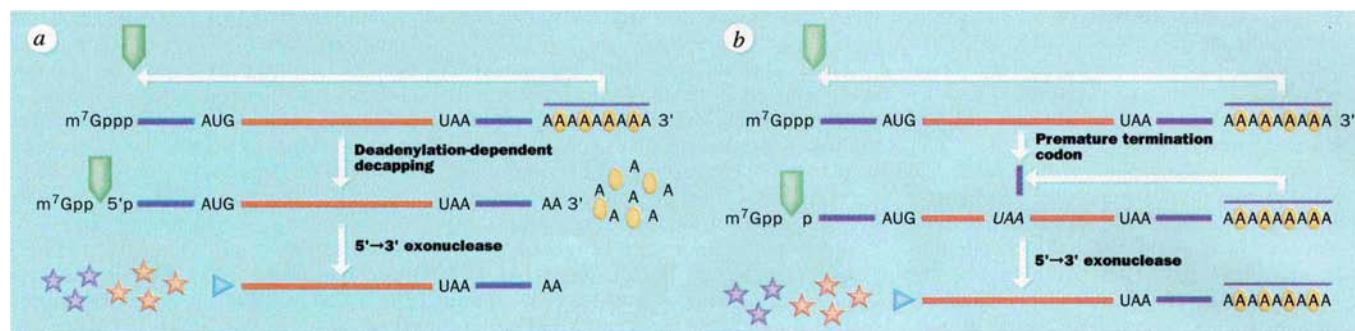


FIG. 1 There are two mRNA degradation pathways in yeast. *a*, A deadenylation-dependent pathway in which, following degradation of the 3' poly(A) tail and loss of the poly(A)-binding protein PABP (yellow), the 5' m⁷G cap is cleaved by the decapping enzyme (green), of which the Dcp1 protein is a component¹. This is followed by rapid degradation of

the body of the mRNA by the 5'→3' exonuclease (blue) which is encoded by the *XRN1* gene. *b*, A deadenylation-independent pathway⁶ that is stimulated by the presence of a translation-termination codon (UAA, shown in *italics*) within the open reading frame of the mRNA. All other components are as in the deadenylation-dependent pathway.

mRNA species? The answer turns out not to be a simple one and involves one or more mRNA-specific sequences and a number of mRNA-binding proteins: some of these are directly responsible for the degradation of mRNA (namely the nucleases), whereas others restrict access of these degradative enzymes to mRNA. For the majority of eukaryotic mRNAs, there are also two natural barriers to mRNA degradation: a 7-methylguanosine (m⁷G) 'cap', which is attached to the 5' end of the mRNA, and a homopolymer of up to 200 adenosine nucleotides — the poly(A) tail — which is added to the 3' end. Both modifications act as barriers to the marauding nucleases that would otherwise rapidly degrade mRNAs nucleotide by nucleotide, beginning either from the 5' end (5'→3' exonucleases) or the 3' end (3'→5' exonucleases) of the molecule.

There are two major mRNA degradative pathways in yeast: those in which the degradation of an mRNA is initiated as a consequence of the loss of all, or almost all, of the poly(A) tail — the so-called deadenylation-dependent pathways (Fig. 1*a*), and those that are activated even in the absence of deadenylation of the mRNA⁵. The archetype of the deadenylation-independent pathways is the nonsense-codon-mediated mRNA-decay

pathway⁶, which is activated by the presence of an aberrantly located translation-termination codon within the normally translated region of an mRNA (Fig. 1*b*). A common element of these two degradative pathways is the *coup de grâce*, the removal of the protective m⁷G cap from the 5' end of the mRNA, which exposes the mRNA to an extremely aggressive 5'→3' exonuclease encoded by the *XRN1* gene⁷. Beelman and colleagues' identification¹ of an essential component of the yeast decapping enzyme as the product of the *DCP1* gene, a protein of relative molecular mass 30,000 designated Dcp1, therefore represents a sizeable step forward in our understanding — not only of how mRNA degradation is initiated, but also why different mRNA species are degraded at vastly different rates.

Yeast cells that have been engineered to lack *DCP1* gene function accumulate deadenylated mRNAs that retain the 5' cap, and the half-lives of a range of mRNAs with different intrinsic stabilities increase significantly¹. That capped but deadenylated mRNAs are still degraded, albeit less efficiently, has important implications because it provides evidence for one or more secondary mRNA-degradative pathways that employ 3'→5' exonucleases to degrade deadenylated mRNAs,

or endonucleases that cut within the body of the mRNA to provide new substrates for the *XRN1* gene-encoded 5'→3' exonuclease. Neither of these minor alternative degradative pathways requires an mRNA to be decapped, which may explain why a *dcp1*-null mutant is viable¹.

That an mRNA is not decapped until the 3' poly(A) tail is significantly reduced in length in the deadenylation-dependent pathway is a remarkable finding, because it demonstrates that events at one end of the mRNA molecule can have a profound influence on events occurring at the other end of the molecule. Clues as to how this long-range interplay can arise have come from the finding that the poly(A) tail binds to a specific protein (the poly(A)-binding protein, or PABP⁸). Decapping can only occur once the poly(A) tail has been shortened to 10–15 residues, at which point PABP can no longer bind (Fig. 1*a*). That mRNAs in *pab1* mutant cells lacking PABP are decapped, even though they possess full-length poly(A) tails⁹, indicates that it is the PABP-poly(A) complex at the 3' end that inhibits decapping at the 5' end of the molecule, and not the poly(A) tail *per se*. A functional interaction between the 5' and 3' ends of an mRNA in the control of maternal mRNA translation and localization in the early embryos of frogs and flies has also been found¹⁰. Remember that the

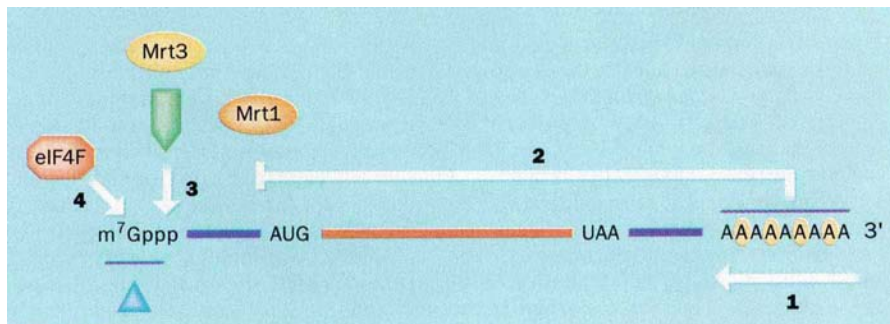


FIG. 2 The intrinsic stability of an mRNA is established by a series of interactions which ultimately influence the rate at which the 5' cap is removed by the decapping enzyme¹. These are: (1) the rate at which the mRNA is deadenylated; (2) the inhibition of the decapping enzyme by the PABP-poly(A) complex; (3) the efficiency with which the decapping enzyme is able to access the 5' m⁷G cap; (4) the ability of the decapping enzyme to compete with the cap-binding translation initiation factor complex eIF4F.

1. Beelman, C. A. *et al.* *Nature* **382**, 642–646 (1996).
2. Brenner, S., Jacob, F. & Meselson, M. *Nature* **190**, 576–581 (1961).
3. Gros, F. *et al.* *Nature* **190**, 581–585 (1961).
4. Herrick, D., Parker, R. & Jacobson, A. *Mol. Cell. Biol.* **10**, 2269–2284 (1990).
5. Caponigro, G. & Parker, R. *Microbiol. Rev.* **60**, 233–249 (1996).
6. Peltz, S. W., He, F., Welch, E. & Jacobson, A. *Prog. Nucleic Acid Res. Mol. Biol.* **47**, 271–298 (1994).
7. Larimer, F. W. & Stevens, A. *Gene* **95**, 85–90 (1990).
8. Sachs, A. B., Bond, M. W. & Kornberg, R. D. *Cell* **45**, 827–835 (1986).
9. Caponigro, G. & Parker, R. *Genes Dev.* **9**, 2421–2432 (1995).
10. Richter, J. D. in *Translational Control* (eds Hershey, J. W. B., Matthews, M. B. & Sonenberg, N.) 481–504 (Cold Spring Harbor Laboratory Press, New York, 1996).
11. Jacobson, A. in *Translational Control* (eds Hershey, J. W. B., Matthews, M. B. & Sonenberg, N.) 451–480 (Cold Spring Harbor Laboratory Press, New York, 1996).
12. Ross, J. *Microbiol. Rev.* **59**, 423–450 (1995).

mRNA molecule is not a straight, linear molecule as depicted in Fig. 1, but rather that the 5' and 3' ends of the mRNA could be physically associated by protein-protein or protein-mRNA interactions to form a 'closed-loop'¹¹.

The realization that the rates of both deadenylation and decapping can contribute to the stability of an mRNA provides us with an insight into what properties of an mRNA molecule set its intrinsic rate of turnover. For example, the sequence and/or structural features of an mRNA may have either a direct or indirect effect on the rate at which the

decapping enzyme is able to bind to the m⁷G cap and cleave it from the mRNA (Fig. 2). The decapping enzyme must also compete with the translation-initiation factor complex eIF4F for the cap. Although the mechanism by which the PABP-poly(A) complex inhibits decapping remains to be elucidated, Beelman *et al.* have also identified two other genes (*MRT1* and *MRT3*) whose products are required for deadenylation-dependent decapping (manuscript submitted).

Although the mist is beginning to clear from the mRNA degradative pathways in yeast, a shroud of it stills lies over the

mammalian pathways. Can we assume that similar, if not identical, pathways exist in mammalian cells? That deadenylation and loss of PABP precedes degradation of mammalian mRNAs¹² would suggest that the answer is yes, but it must now be established how the mRNA is then degraded. Undoubtedly, even as you read this article, PCR machines are churning out *XRN1* and *DCP1* homologues from higher eukaryotic cells. □

Mick F. Tuite is in the Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK.

NEUROBIOLOGY

Tales of the unexpected



MONKEYS, like humans, do not take kindly to having their hopes raised only to be dashed, as illustrated in this cartoon. It depicts a classic experiment, published by O. L. Tinklepaugh in 1928, in which a monkey is shown a food reward that is then concealed. The monkey must remember the location of the reward for several seconds before receiving a cue to uncover and collect it. The monkey prefers banana, but will settle for lettuce; if, however, it expects banana but finds lettuce instead, it becomes angry, indicating that it remembers not only the location but also the nature of the expected reward.

On page 629 of this issue, Masataka Watanabe provides new insight into

how the brain stores such expectations, by recording from single forebrain neurons as monkeys perform delay-reward tasks similar to Tinklepaugh's. In some cases, the monkey could actually see the food reward in advance, while in others its location was cued by a light, and only from previous trials does the monkey know which reward to expect. It is known that neurons in the dorsolateral prefrontal cortex store information about location of hidden cues; this so-called delay activity is thought to underlie short-term spatial working memory. Watanabe now shows, however, that these neurons also store information about the nature of the expectation.

Some neurons fire selectively in antic-

ipation of a particular type of reward (for instance, cabbage versus raisin), some are selective for its location (left versus right), and some are influenced by whether the reward has actually been seen rather than merely inferred from experience. It is possible to see how learning affects their activity; Fig. 4b of the paper (page 631) illustrates how one neuron's response to a light cue changes as the monkey realizes that the cue now predicts potato rather than grape juice. The combined activities of many such neurons could in principle encode a detailed description of the expected reward, which could then be used to control goal-directed behaviour.

Charles Jennings

Nanotechnology and nucleotides

Donald Bethell and David J. Schiffrin

THERE are many advantages to making things small, such as the potential speed and compactness of nanometre-scale electronic components, and the remarkable, size-dependent physical properties that very small particles can have. Building complicated structures on this scale isn't easy, but the problem is being tackled from several directions. Mucic *et al.*¹ and Alivisatos *et al.*² (respectively pages 607 and 609 in this issue) now describe how they have used oligonucleotides to organize metal clusters in superlattices, and produced materials that are a halfway house between simple synthetic structures and biological materials.

Michael Faraday, in the 1856 Bakerian Lecture³, observed that "gold wire deflagrated by explosions of a Leyden battery produces a divided condition, very different to that presented by gold leaves." The ruby colour of the deposit observed on transmission could be transformed to the green colour of gold leaves by applying a compressive force. This was, perhaps, the first observation that the properties of very small particles can differ from those of the bulk material, and that the control of their separation and coalescence leads to profound changes in their properties.

More recently, self-organization of nanoparticles has promised a powerful route to new materials, in particular the synthesis of superlattices — solid, periodic arrays of structures having different electronic properties^{4,5}. A possible way to achieve this is to prepare the nanosized components of the three-dimensional structure by colloidal chemistry, and then use self-assembly as a way of joining them together. The difficulty with this approach is that imperfections in the superlattice can result from inadequate chemical recognition by the constituents. This can be a considerable limitation in making nanostructured materials for electronic applications, where long-range order is important.

Biological systems must be able to cope with complex recognition problems, and DNA in particular is suited to transmit well-defined chemical information, making use of the pairing properties of nucleotide bases. Mucic *et al.*¹ and Alivisatos *et al.*² report their initial successes in this form of self-assembly using inorganic particles, in this case colloidal gold, using base-pairing between complementary chains of attached oligonucleotides to provide the link between the particles. Because of the specificity of base-pairing, this procedure in principle allows complex patterns of interlinked gold particles with predetermined separations to be assembled using a standard technique.

The binding is noncovalent and so the

linking can probably be readily reversed. That could allow complex networks of particles capable of molecular recognition and signal transduction by virtue of having variable electronic properties. Such materials could find application in biosensors.

The organization of pre-fabricated

IMAGE
UNAVAILABLE
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REASONS

An early nanotechnologist — Faraday was the first to study metal colloids.

structures has been attempted before by ordinary self-assembly techniques, using solutions of colloidal nanoparticles as the starting point and two-ended linker molecules for building up superlattices⁶. Such organic synthetic methods can be devised to be highly specific, but they require a range of functional groups and reaction conditions that do not have the simplicity offered by this new use of oligonucleotides. They do, however, offer the possibility of varying the physical properties of the link by the inclusion of donor or acceptor moieties at specific points within the superlattice, for example.

The attachment of reactive groups to clusters is another important approach in attempts to build structures of well-defined electronic properties from small building blocks. For example, in a recent publication Royce W. Murray and co-workers⁷ have shown that thiol-stabilized nanoclusters can be used as a starting point for further modification by substituting individual ligands with thiols that have functional groups at the end of their hydrocarbon chain. These are capable of chemical reaction to produce two- and three-dimensional networks. The materials produced in this way can best be described as intermediate in nature between a continuous material and an organic molecular solid.

The synthesis of new nanostructured

materials requires homogeneity in particle size. Leff *et al.*⁸ and Whetten *et al.*⁹ both used classical preparation and separation methods such as fractional precipitation to achieve size control of metal particles bearing self-assembled monolayers. The latter group has isolated nanocrystals of precise sizes, determined by the 'magic numbers' that relate surface area to volume and correspond to local energy minima. Furthermore, the crystallization of these materials into two-dimensional superlattices has been demonstrated.

All of these discoveries must be viewed against the background of attempts to synthesize new materials with unusual optical and electronic properties. There is at present a confluence of technological developments and basic science that has a good chance of creating controlled structures that can lead to new electronic devices. An example of these incipient technologies is the work of Y. Wada and colleagues on nanoscale devices¹⁰ — they are attempting to make ultra-fast switches by arranging atoms or molecules on a surface one by one, using a scanning tunnelling microscope (STM). This same group has now produced a micromechanical STM on a chip¹¹, using the electronic techniques originally developed to make high-capacity memory chips. The dramatic cost reduction that can be envisaged in such devices will make it practical to manipulate clusters, or individual atoms, for the manufacture of ultraminiaturized electronic circuits.

The work now described by Mucic and Alivisatos is a major development in that it refines the control of supramolecular self-assembly. These and other recent advances in the synthesis of nanosized components that can be handled like simple organic compounds, and in the methods of self-assembly of structures, are all leading towards fabrication of electronic components on a molecular scale. The worldwide effort in this and related areas will surely lead to exciting developments in new electronic materials over the next ten years. □

Donald Bethell and David J. Schiffrin are in the Department of Chemistry, University of Liverpool, Liverpool L69 3BX, UK.

- Mucic, R. C., Storhoff, J. J., Letsinger, R. L. & Mirkin, C. A. *Nature* **382**, 607–609 (1996).
- Alivisatos, A. P. *et al.* *Nature* **382**, 609–611 (1996).
- Faraday, M. *Phil. Trans. R. Soc. Lond.* **147**, 145–181 (1857).
- Braun, P. V., Osenar, P. & Stupp, S. I. *Nature* **380**, 325–328 (1996).
- Weller, H. *Angew. Chem.* **35**, 1079–1081 (1996).
- Bethell, D., Brust, M., Schiffrin, D. J. & Kiely, C. *J. Electroanal. Chem.* **409**, 137–143 (1996).
- Hostetter, M. J., Green, S. J., Stokes, J. J. & Murray, R. W. *J. Am. Chem. Soc.* **118**, 4212–4213 (1996).
- Leff, D. V., Ohara, P. C., Heath, J. R. & Gelbart, W. M. *J. Phys. Chem.* **99**, 7036–7041 (1995).
- Whetten, R. L. *et al.* *Adv. Mater.* **8**, 428–433 (1996).
- Wada, Y., Uda, T., Lutwyche, M., Kondo, S. & Heibe, S. *J. Appl. Phys.* **74**, 7321–7328 (1993).
- Lutwyche, M. I. & Wada, Y. *Sensors Actuators A* **48**, 127–136 (1995).

Southern discomfort

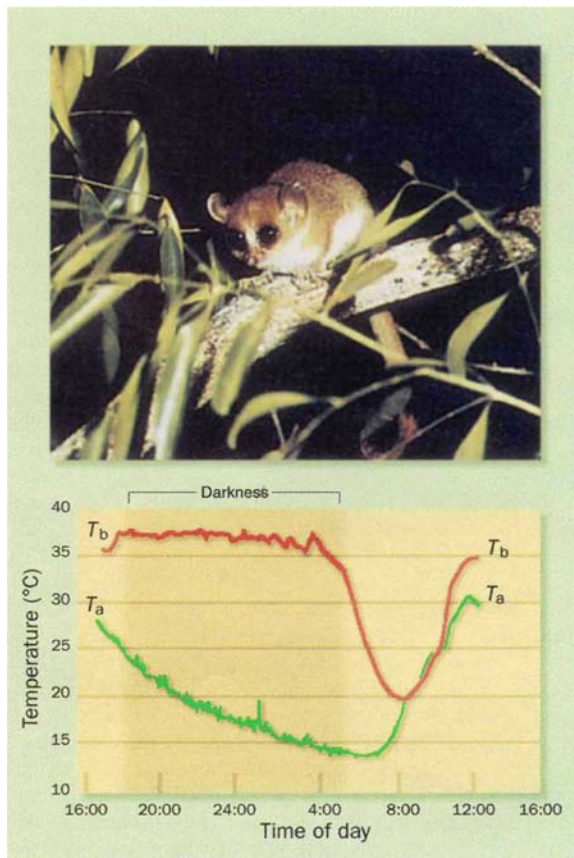
Andrew R. Cossins and Brian Barnes

WE all know that winter hibernation, when body temperature plunges from the normal 37 °C to as low as -2 °C, allows some specialized mammalian species to survive particularly severe winter conditions. Metabolism is depressed to low, energy-saving levels until the spring, when activation of the animal's brown adipose tissue (the heat-producing fat cells found in small mammals and in the young of larger mammals) stokes up body temperature in time for mating. But this traditional northern hemispheric view was up-ended at a conference held in Australia last month*, when it was acknowledged that a surprisingly large proportion of mammals from down under, including a monotreme (the echidna) and various marsupial and placental species, routinely enter daily torpor and seasonal hibernation, often under surprisingly mild conditions. What is more, when the marsupials arouse and rewarm, it is apparently without the benefit of brown adipose tissue.

The number of species known to undergo torpor and hibernation is being constantly revised upwards with the continuing improvements in remote radiosensing as a technique for measuring body temperature. Newly discovered heterotherms include the mountain pigmy possum, which has an extended five-month hibernation in the Australian Alps at a body temperature that goes down to 2 °C (F. Geiser, Univ. New England, Armidale, Australia), and two species of mouse lemur from Madagascar, not a climate known for extreme cold, which show a pronounced daily torpor during the seasonally dry winter period (see figure), with body temperatures of less than 10 °C (G. Heldmaier, Univ. Marburg, Germany). No doubt this list will grow ever longer, especially as the new implantable digital data-loggers come into routine use for accumulating the thermal experiences of entirely free-ranging animals.

Just why so many Southern Hemisphere species show torpor is not clear. Marsupials and monotremes have lower basal metabolic rates and body temperatures than typical placental (northern) mammals. However, some placental mammal species

from southern zoogeographical zones also have low basal metabolic rates (B. Lovegrove, Univ. Natal, Scottsville, South Africa). This, and the increased prevalence of torpor in southern mammals, is now attributed to climatic unpredictability, especially the periodic dry spells caused by El Niño ocean-circulation events.



Top, the western rufous mouse lemur, *Microcebus myoxinus*, believed to be extinct but recently rediscovered in forests of Madagascar. Bottom, the changes in ambient temperature (T_a) and body temperature (T_b) of lemurs over a 24-hour period, during which torpor was evident. T_b was kept high during the dark feeding period, despite a progressive drop in T_a . T_b dropped in the morning to 20 °C and then recovered by mid-afternoon. The early phase of warming appears to rely on the increasing daytime ambient temperature, the response giving a considerable energetic saving over sustained homeothermy. (Data from S. Ortmann, J. Schmidt, U. Ganzhorn & G. Heldmaier *Naturwissenschaften*, in the press.)

Hypothermia and the reduced demand for energy improves survival during these stressful periods of reduced food availability. So the commonly accepted view that the low body temperature of marsupials and monotremes results from their primitive thermoregulatory status should be replaced by a recognition of their climatic adaptation. Thus, the echidna is well insulated and copes well with cold temperatures, but it hibernates in comparatively mild conditions, even when food is readily

available (S. Nicol, Univ. Tasmania).

In some mammals, the period immediately preceding hibernation is characterized by a massive increase in food intake and a doubling of body weight as fat is accumulated to survive the long period of torpor. Administration of leptin (the newly discovered satiety hormone produced by white fat) to arctic ground squirrels during this fattening phase curbs their appetite and significantly reduces body weight and fat deposits (B. B. Boyer, Inst. Arctic Biology, Fairbanks). This pronounced seasonal cycle of extreme weight gain and loss offers a potentially tractable model for a more general understanding of how the brain's regulation of feeding behaviour balances food intake with energy expenditure over the longer term. Interestingly, hibernation is sensitive to the quality of lipid diet, with polyunsaturated fats enhancing both the length and depth of torpor (C. Frank, Fordham Univ., New York), and involves substantial changes in the expression of lipid biosynthetic enzymes (G. Florant, Colorado State Univ., Ft Collins; R. Coleman, Univ. North Carolina, Chapel Hill).

The monotreme and marsupial hibernators show all the features of classical seasonal hibernation, including the dramatic reduction in heat production on entry into torpor and the periodic arousals to normal body temperatures (S. Nicol, Univ. Tasmania; G. Grigg, Univ. Queensland, Brisbane; P. D. Rismiller, Univ. Adelaide). However, they do not possess the brown adipose tissue of small placentals which, being under the control of the autonomic nervous system, produces heat during arousal.

This highlights a general lack of understanding of thermoregulatory heat production in tissues other than brown adipose, and species without this specialized brown fat offer good models for exploring alternative methods of thermogenesis. Hindlimb preparations from rat, chicken and a small wallaby-like marsupial, the bettong, increase their oxygen consumption (and heat production) in response to an infusion of noradrenaline or other vasoconstrictors by switching on capillary perfusion through metabolically active regions (M. G. Clark, Univ. Tasmania).

It is not yet clear whether this heating response has the necessary power and cold-inducibility to explain either arousal or thermoregulation in the cold. Noradrenaline, which mediates the autonomic activation of heat production in placental mammals, has no thermogenic effect in echidnas or in many marsupial groups,

*Adaptations to the Cold, 10th International Hibernation Symposium, Cradle Mountain Lodge, Tasmania, Australia, 30 June–6 July 1996. Contributions published in *Adaptations to the Cold* (eds Geiser, F., Hulbert, A. J. & Nicol, S. C.) (Univ. New England Press, Armidale, Australia, 1996).

including those that show torpor and hibernate, so the autonomic system probably does not mediate arousal in these species.

Birds also lack brown adipose tissue, but produce thermoregulatory heat in the cold by increased tone of antagonistic muscles. Activation of increasing numbers of skeletal muscle fibres matches heat production to the thermoregulatory needs of the animal (E. Hohtola, Univ. Oulu, Finland) until muscle tremor or shivering becomes evident. Heat production by vigorous shivering is certainly involved in arousal of some small marsupial hibernators (F. Geiser), but it remains to be seen whether muscle tone is modulated as it is with birds.

Brown adipose tissue is thus seen as a specifically placental innovation which reduces dependence on muscle activation for thermoregulation in the cold and during arousal from low body temperature. Its restriction to a single endothermic group is puzzling, but one novel suggestion is that it aids thermoregulation in neonates

(babies) abandoned by actively foraging parents (B. Wunder, Colorado State Univ., Ft Collins). This is, of course, not a problem for the marsupial mammals.

Despite all this, there is little progress in two long-standing and unresolved problems in hibernation research. First, why do hibernating mammals of all types wake up at regular intervals for brief periods during hibernation? Second, how do hibernators survive such low body temperatures, which would kill non-hibernating species? Given that these questions profoundly affect our understanding of the non-hibernating state, answers might best come from those not currently involved in hibernation research. □

Andrew R. Cossins is in the Department of Environmental and Evolutionary Biology, University of Liverpool, Liverpool L69 3BX, UK. Brian Barnes is at the Institute of Arctic Biology, University of Alaska, Fairbanks, Alaska 99775, USA.

ZEOLITES

Design for sieving

Mark E. Davis

ZEOLITES and zeolite-like molecular sieves are fascinating materials from both an intellectual and a practical standpoint. Their elegant crystal structures have voids with precise and uniform sizes, in the range of about 0.4–1.5 nm, which can be used for molecular sieving (that is, to discriminate between molecules of similar size). Zeolites are used as shape-selective catalysts — they can encourage reactions that favour one particular product over another even when the difference in size between the two species is as little as 0.03 nm, as is the case for *p*-xylene and *o*-xylene. Further, zeolites are environmentally benign solids. So their use as catalysts continues to burgeon. But in order to target a particular reaction of interest, can zeolites be synthesized by rational design? Currently, the answer is no, but great progress in this area is underway. Lewis and colleagues, on page 604 of this issue¹, provide the first computational scheme for generating organic molecules that are candidates for structure direction in zeolite synthesis.

Zeolites that contain a high fraction of silicon dioxide ($\text{SiO}_2/\text{Al}_2\text{O}_3 > 40$) are hydrophobic, and can be prepared using organic molecules that direct the crystallization pathway. These organic species become enclathrated (trapped in the zeolite cavity) during the synthesis to form an organic-inorganic composite, and their removal generates the void. These organic molecules are often called templates. But the inorganic zeolite material does not follow the organic structure closely enough for this to be true templating². So I will

refer to these molecules as structure-directing agents (SDAs) since they determine the outcome of zeolite synthesis.

High-silica zeolites can adopt many structures that are energetically similar to quartz³ (differing by less than 15 kJ mol⁻¹). Knowing the phase diagram of silica alone, however, will not help in the design of zeolite synthesis, because zeolites are metastable phases that are produced via kinetic pathways⁴. A thermodynamic analysis of a complete zeolite synthesis mixture⁵ shows that there is both an enthalpic and an entropic driving force. That is, SDAs that become hydrophobically hydrated entities in water provide two forces that drive crystallization — an enthalpic force because the SDA would rather be enclathrated in the hydrophobic silicate than in water; and an entropic force because release of the hydration sphere reduces overall order^{4,5}. In this way, the hydroxide of the tetrapropylammonium cation can be used to convert quartz into a tetrapropylammonium-containing zeolite named ZSM-5.

In principle, the design of SDAs could lead to the rational design of high-silica zeolites, and Lewis *et al.* provide the first methodology for automatically searching for appropriate SDAs, using a computer model to 'grow' organic molecules until they fit the desired void (see Fig. 2 on page 605). This approach is particularly appealing as there are also many known structures for which new and less expensive SDAs could allow commercialization⁶. Furthermore, hundreds of theoretical

structures can be generated using simulated annealing techniques⁷, and the combination of the methodology of Lewis *et al.* with structure generation could provide real leads to the design of new zeolites. One particularly interesting problem is the synthesis of a chiral zeolite, which could distinguish between left- and right-handed molecules. The use of computational power outlined by Lewis and colleagues could greatly speed up the search for chiral SDAs using theoretical, chiral zeolite structures as models^{2,8}.

One may argue about the precise force fields used by Lewis *et al.* and the lack of interactions between the organic and the zeolite other than van der Waals forces, but the method is probably good enough for modelling pure-silica zeolites (where Si–O⁻ defect sites balance the charge⁹ on each SDA, and van der Waals interactions are the controlling factors). More sophistication can be incorporated later. What is exciting about this work is that many different organic molecules are automatically generated. Although the hydrocarbon species are not likely to function as SDAs owing to their low solubility in water, most synthetic chemists could easily make the conceptual leap from the hydrocarbon to a water-soluble species, perhaps by the inclusion of an amine, as Lewis *et al.* did for the synthesis of DAF-4, or the substitution of a nitrogen for a quaternary carbon.

Lewis *et al.* use both periodic and non-periodic boundary conditions in their ZSM-5 example. Although they favour the periodic (modelling a single zeolite unit cell, in other words) to inhibit molecular growth, I suggest that the non-periodic method not be abandoned. Several organic molecules that span more than one channel intersection can be used to crystallize ZSM-5, and some of them mimic two of Lewis and colleagues' candidate templates. This is an interesting feature of the new results and could be particularly important in designing new SDAs to control the stacking sequence of layers in the growth of some high-silica zeolites⁴. Controlling the layer sequence of zeolite-β, an intergrowth of two structures, A and B, could produce a pure chiral version of polymorph A^{2,7}.

Given the advances in the understanding of zeolite crystallization and the com-

1. Lewis, D. W., Willock, D. J., Catlow, C. R. A., Thomas, J. M. & Hutchings, G. J. *Nature* **382**, 604–606 (1996).
2. Davis, M. E. & Lobo, R. F. *Chem. Mater.* **4**, 756–768 (1992).
3. Petrovic, I., Navrotsky, A., Davis, M. E. & Zones, S. I. *Chem. Mater.* **5**, 1805–1813 (1993).
4. Davis, M. E. *Stud. Surf. Sci. Catal.* **97**, 35–44 (1995).
5. Helmkamp, M. M. & Davis, M. F. *Annu. Rev. Mater. Sci.* **25**, 161–192 (1995).
6. Freyhardt, C. C., Tsapatsis, M., Lobo, R. F., Balkus, K. J. Jr & Davis, M. E. *Nature* **381**, 295–298 (1996).
7. Deem, M. W. & Newsam, J. M. *J. Am. Chem. Soc.* **114**, 7189–7198 (1992).
8. Treacy, M. M. J. & Newsam, J. M. *Nature* **332**, 249–251 (1986).
9. Koller, H., Lobo, R. F., Burkett, S. L. & Davis, M. E. *J. Phys. Chem.* **99**, 12588–12596 (1995).

putational tools being developed, is the design of a zeolite any closer to becoming reality? In my opinion the answer to this question is yes. I feel that there remains a critical question, however, that must be answered first: what controls the kinetics of zeolite nucleation? After all, this is a kinetically controlled process, and in high-

silica synthesis it is usually the structure that nucleates first that is ultimately produced. □

Mark E. Davis is in the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, USA.

FAT METABOLISM

Slimming with a leaner enzyme

Bradford B. Lowell

AFTER a hormone binds at the cell surface, resulting in the generation of the messenger molecule cyclic AMP inside the cell, nearly all of the subsequent actions are mediated by cAMP-dependent protein kinase (PKA). Genes encoding four different types of regulatory subunits (RI α , RI β , RII α and RII β) that form part of this enzyme have been identified¹, but the functional significance of each with respect to the others was unknown. On page 622 of this issue, Cummings *et al.*² describe the features of gene-knockout mice specially bred to lack the gene encoding RII β . RII β is heavily expressed in brain and in fat cells (of which there are two types — white and brown adipocytes), where it is normally the principal R subunit. Although the loss of RII β is partially compensated for by an increase in one of the other R subunits (RI α), surprisingly, the PKA activity in brown adipocytes of these mice was stepped up, giving them increased energy expenditure, leanness and a marked resistance to diet-induced obesity.

PKA is a tetrameric protein composed of two monomeric catalytic (C) subunits which will phosphorylate the amino-acid serine (and occasionally threonine) on other proteins, and a dimeric regulatory (R) subunit that prevents the enzyme from working when cAMP is scarce³ (Fig. 1). The multiplicity of R and C isoforms, each encoded by unique genes, suggests that isoform-specific functional differences may exist^{1,3,4}; C isoforms seem to be similar, including their specificity for substrates, but RI and RII isoforms are functionally distinct. Features common to all R isoforms include a homodimerization domain, two tandem cAMP-binding domains, and an autoinhibitory domain which blocks the kinase activity of C isoforms when cAMP is not bound. RII, but not RI, isoforms are phosphorylated in the autoinhibitory domain by C, reducing the affinity of R for C. In addition, R isoforms may 'fine-tune' cAMP sensitivity of the PKA holoenzyme. Indeed, such an effect was found in the mice of Cummings *et al.*², where loss of RII β in brown fat induced compensatory upregulation of RI α , switching tetrameric PKA from RII β ₂C₂ to RI α ₂C₂. The mutation

resulted in more avid cAMP binding, greater cAMP sensitivity and increased kinase activity, demonstrating that RI α and RII β are functionally distinct.

An important feature of R isoforms is that they determine the subcellular localization of PKA: RI-PKA is primarily cytoplasmic, whereas most RII-PKA is associated with the plasma membrane, subcellular organelles and the cytoskeleton. Compartmentalization of PKA is due to high-affinity binding of RII subunits to A-kinase anchoring proteins, which in turn are targeted to various sub-

stream targets of PKA include hormone-sensitive lipase, which breaks down triglyceride in fat stores to release free fatty acids, and CREB (for cAMP-response-element-binding protein), which stimulates transcription from the UCP gene^{8,9}.

It is interesting that the dominant substrate for PKA in white, and probably also brown, adipocytes is perilipin¹⁰, an abundant protein of unknown function found on the surface of triglyceride droplets. Given its location, abundance and dense phosphorylation, perilipin is thought to be involved in PKA-mediated activation of lipolysis, possibly by modulating access of hormone-sensitive lipase to the triglyceride droplets. Other, less well understood but equally important consequences of PKA activation in brown adipose tissue include increases in mitochondrial biogenesis and in brown adipose tissue growth and differentiation, and stimulation of type-II thyroxine deiodinase activity, which increases intracellular concentrations of the hormone triiodothyronine to stimulate UCP gene transcription.

In RII β -knockout mice², chronic PKA 'overactivity' in brown fat stimulated whole-body energy expenditure, and mice lost fat while essentially normal food

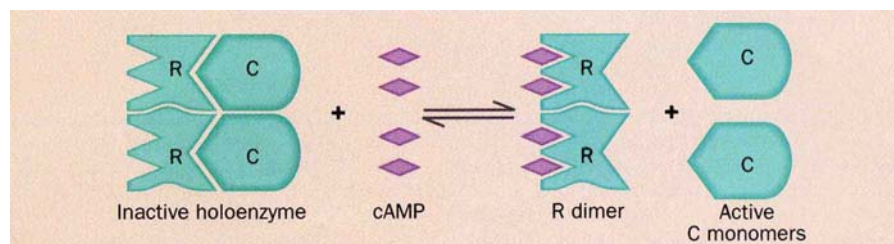


FIG. 1 Regulation of protein kinase A by cyclic AMP. Two cAMP molecules bind to each regulatory subunit (R), decreasing the affinity of this subunit for the catalytic subunit (C) by 10,000–100,000-fold. Tetrameric PKA dissociates into two C monomers and dimeric R, releasing C from inhibition by R.

cellular sites⁵. This anchors PKA close to critical targets and/or 'co-anchors' PKA with inactivating dephosphorylating enzymes such as protein phosphatase 2B (ref. 6), tightly regulating the phosphorylation status of critical substrates. Although the role of the A-kinase anchoring proteins and compartmentalization of PKA in brown adipocytes is unknown, the isoform switch from type II to type I in RII β -deficient brown fat is likely to cause subcellular redistribution of PKA, possibly contributing to PKA dysregulation.

Brown fat is an important site of adrenaline-stimulated energy expenditure⁷ (Fig. 2). Central to this response is uncoupling protein (UCP), an inner-membrane mitochondrial protein found only in brown-fat adipocytes, which exports fatty acid anions, thus uncoupling mitochondrial respiration from ATP production. Noradrenaline released from sympathetic nerve endings stimulates two different types of receptors at the cell surface, α - and β -adrenoceptors, the latter causing increased cAMP and PKA activation. Key down-

intake was maintained. Also, mutant mice were completely resistant to obesity induced by a high fat diet and the development of fatty livers. Note that this leanness in mutant mice was associated with a marked reduction in levels of messenger RNA and circulating leptin, the adipocyte-secreted satiety factor absent in *ob/ob* mice¹¹. As leptin deficiency in *ob/ob* mice causes increased food intake, it is unexpected that RII β -deficient mice, with low leptin levels, do not have compensatory hyperphagia — perhaps there is a satiety factor that counters the effects of low leptin. This satiety signal could emanate from stimulated brown fat, or possibly from the brain, where RII β deficiency might also be expected to cause PKA dysregulation.

Of note, another study found that mice treated with a highly selective β_3 -adrenoceptor agonist, which specifically activates PKA in both white and brown fat, have reduced triglyceride stores, low leptin levels and decreased food intake¹², again opposite to what might be predicted from

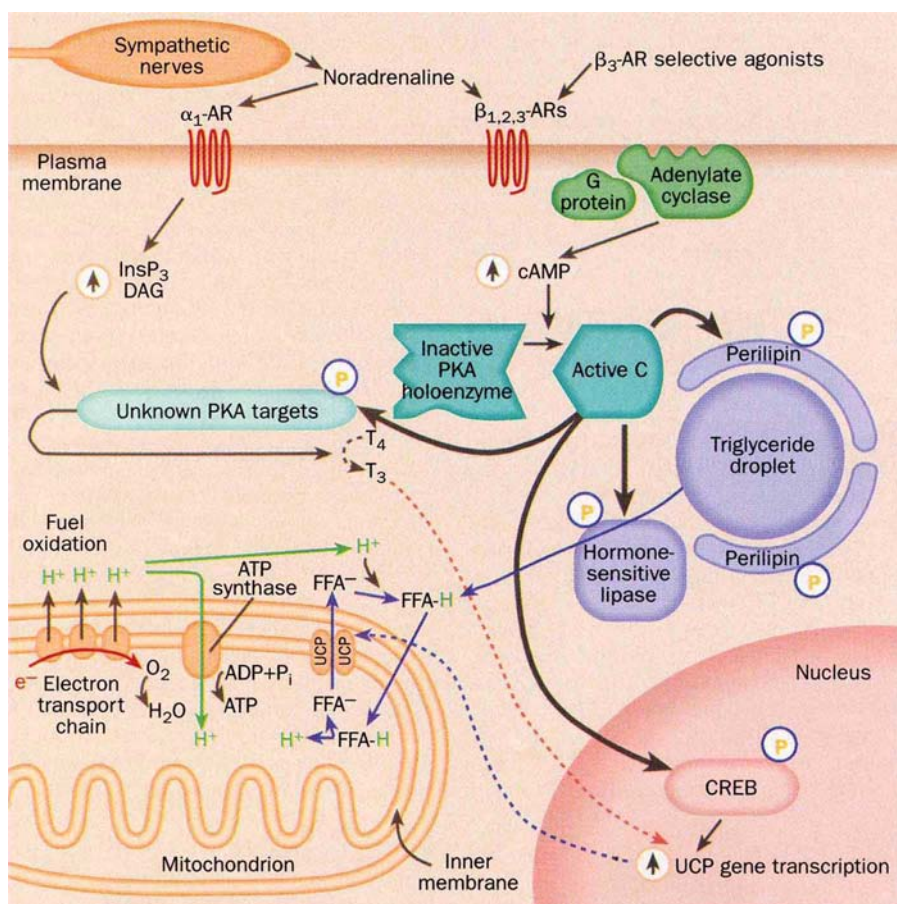


FIG. 2 Adrenergic control of uncoupled respiration in brown adipocytes. Fuels are oxidized by means of the electron transport chain in mitochondria, creating a proton electrochemical potential gradient. Normally, protons descend this gradient via ATP synthase, coupling synthesis of ATP to fuel oxidation. However, if no work has been done, little ADP exists and protons cannot re-enter via ATP synthase. The proton gradient increases, electron transport slows and fuel oxidation stops. Uncoupling protein (UCP) causes the proton gradient to collapse by exporting free fatty acid (FFA) anions which then function as cycling proton carriers¹⁶. In the uncoupled state, brown adipocytes oxidize fuels unrelated to the performance of work, the by-product being heat. Release of noradrenaline from sympathetic nerve endings or treatment with β_3 -adrenoceptor agonists initiates uncoupled respiration by activating PKA, which stimulates hormone-sensitive lipase to provide the necessary FFAs and phosphorylates CREB to increase UCP gene transcription. Other equally important events that are less well understood are described in the text. AR, adreno(re)ceptor; InsP₃, inositol trisphosphate; and DAG, diacylglycerol (the latter are signalling molecules).

low leptin levels. Finally, transgenic mice with diminished brown fat have greatly increased triglyceride stores, high leptin levels and, unexpectedly, normal or increased food intake^{13,14}. Taken together, these observations raise the possibility that brown fat generates a satiety signal that antagonizes the effects of low and high leptin levels.

The leanness of RII β -knockout mice highlights the capacity of PKA and its downstream targets in adipocytes to regulate body fat content, so mutations affect-

ing this pathway could well contribute to human obesity. Furthermore, drugs that stimulate PKA, such as β_3 -adrenoceptor agonists¹⁵, might prove useful as anti-obesity drugs — only time and further investigation will tell whether the PKA pathway leads to that Holy Grail. □

Bradford B. Lowell is in the Division of Endocrinology, Department of Medicine, Beth Israel Hospital and Harvard Medical School, 330 Brookline Avenue, Boston, Massachusetts 02215, USA.

DAEDALUS

Central lighting

MODERN optical fibres, with their vast bandwidths and extremely low losses, have transformed communications. Daedalus has a far more obvious use for them. He wants them to transmit light. At present we generate electricity in big stations, send it along many lossy cables, and use it to power millions of inefficient little lamps around the cable network. It would be far better to generate 'central light' in a few big, efficient lamps, and to distribute it almost losslessly by fibre optics.

The snag is switching. A fibre-optic lamp would take the form of a loop in the cable, perhaps descending from the ceiling of the room it has to light. Switching it on would bleed a small fraction of the light from the loop; switching off would allow all the light to pass on to consumers downstream. But how to do this? One way might be to immerse the loop in a jar of glycerine, when total internal reflection in the fibre would be frustrated, and a little light would leak out. But Daedalus reckons that optic-fibre illumination is best suited to public and street lighting, where switching can be done centrally.

The system could be very simple. A few enormous, efficient discharge lamps will be totally surrounded by the close-packed ends of optical fibres, which will capture all the light of each lamp. Each type of discharge lamp emits its own horrid monochromatic colour, but a fairly balanced white light could be created by intertwining fibres from several different types of lamp. These mixed-fibre bundles will run under the pavements and along the highways of the region, just as electricity cables do now. They will curl over from the tops of lamp standards to shine down on the street below. No light will be wasted upwards. Even better, one fibre bundle can be subdivided into vast numbers of single-fibre point sources. These could be arrayed around the fabric of the region, picking out road markings, highlighting public notices and traffic signs in dot-matrix letters, or outlining low bridges and the risers of steps and stairways. By picking out fibres from specific master lamps, they could even be colour-coded. The system would light a city or region far more usefully than at present, and on far less power.

Daedalus even plans to put an audio or radio modulation on the public light. This would be far too fast to be perceptible, of course; but a button-hole photodiode driving a simple receiver could pick up municipal messages about traffic conditions, forecasted weather, local events, and so on. The citizens would be illuminated in more ways than one.

David Jones

- McKnight, G. S. *Curr. Opin. Cell Biol.* **3**, 213–217 (1991).
- Cummings, D. E. *et al. Nature* **382**, 622–626 (1996).
- Francis, S. H. & Corbin, J. D. *Annu. Rev. Physiol.* **56**, 237–272 (1994).
- Daskeland, S. O., Maronde, E. & Gjertsen, B. T. *Biochim. Biophys. Acta* **1178**, 249–258 (1993).
- Scott, J. D. & McCartney, S. *Mol. Endocrinol.* **8**, 5–11 (1994).
- Coghlan, V. M. *et al. Science* **267**, 108–111 (1995).
- Himms-Hagen, J. *Prog. Lipid Res.* **28**, 67–115 (1989).
- Kozak, U. C. *et al. Mol. Cell. Biol.* **14**, 59–67 (1994).

- Cassard-Doulcier, A.-M. *et al. J. Biol. Chem.* **269**, 24335–24342 (1994).
- Greenberg, A. S. *et al. J. Biol. Chem.* **266**, 11341–11346 (1991).
- Zhang, Y. *et al. Nature* **372**, 425–432 (1994).
- Mantzoros, C. S. *et al. Diabetes* **45**, 909–914 (1996).
- Lowell, B. B. *et al. Nature* **366**, 740–742 (1993).
- Frederich, R. C. *et al. Nature Med.* **1**, 1311–1314 (1995).
- Himms-Hagen, J. & Danforth, E. Jr *Curr. Opin. Endocrinol. Diabetes* **3**, 59–65 (1996).
- Jezeq, P., Hanus, J., Semrad, C. & Garlid, K. D. *J. Biol. Chem.* **271**, 6199–6205 (1996).

Bracken ptaquiloside in milk

SIR — Ptaquiloside, a norsesquiterpene¹ found in bracken (*Pteridium aquilinum*), is a potent carcinogen² that binds covalently to nucleotides, causing the splitting of uncoiled DNA³. Its potential for causing tumours and other ailments in farm animals is well established⁴. The observation that milk from cows feeding on a diet containing bracken fronds is carcinogenic and mutagenic to mice and rats^{5,6} led to the suspicion that a bracken compound was present in this milk. When ptaquiloside was first discovered, it was the chief candidate for this carcinogen, but has never been demonstrated to be present in milk. Here we report our attempts to ascertain its presence in milk, to estimate the relationship between the amount of ptaquiloside ingested by cows via bracken and the amount excreted through milk, and to assess the risk of drinking raw milk from cows exposed to bracken. We find that ptaquiloside is excreted in milk at a concentration of about $8.6 \pm 1.2\%$ of the amount ingested by the cow, and is linearly dose-dependent.

We used milk from six two-year-old cows from an agricultural research station

in Mérida, Venezuela, with an average milk production of 20 ± 2 litres per day. For five consecutive days we gave the cows artificial diets containing 6.0 kg per day (2,400 to 10,000 mg per animal per day of ptaquiloside) of previously analysed⁷ freshly collected young bracken fronds (neotropical var. *caudatum*). Milk samples were drawn each morning, frozen and analysed immediately⁸ until all ptaquiloside disappeared. None of the cows showed signs of intoxication during treatment.

We detected and quantified unstable, water-soluble ptaquiloside by selectively extracting it from deproteinized, defatted milk as stable pterosin B, the only alkali-induced decomposition product of ptaquiloside. Pterosin B does not occur naturally in milk from bracken-fed cows, and appears only after changing the pH of the defatted milk to 11 for 2 h at 40 °C. The per cent conversion curves of ptaquiloside in milk from bracken-fed cows and artificially ptaquiloside-contaminated milk in alkali at 38 °C overlap strikingly well over the 70 min of this experiment. We found no other secondary metabolite of bracken that could produce pterosin B under these mild conditions. Thus, pterosin B represents only its precursor, ptaquiloside. We performed quantitative assessment by dichloromethane extraction and reverse-phase HPLC analysis against standards. We also identified pterosin B in milk extracts by comparison of retention times in three mobile phases with pure compound and by the exact coincidence of the ultraviolet spectrum of the suspected peak ($\lambda_{\max} = 218, 260$ nm) by stopped-flow scanning of the eluate.

We detected ptaquiloside in milk for the first time 38 h after feeding cows with bracken fronds. At a constant intake, the amount of ptaquiloside excreted daily increased progressively until the fourth day when it levelled off (*a* in the figure). After day five, ptaquiloside continued to be excreted at approximately the same rate for 62 h after the last treatment. Then, it decreased rapidly and disappeared from the milk by 86 h after the last intake. The average value of total ptaquiloside excreted $[\text{ptaq}]_e$ in milk is $8.6 \pm 1.2\%$ of the total ptaquiloside ingested $[\text{ptaq}]_i$ over the entire feeding period. The daily $[\text{ptaq}]_e / [\text{ptaq}]_i$ ratio during the first 38 h of feeding was independent of the dose ingested. However, there was a linear dependency of $[\text{ptaq}]_e$ on the dose given to the animal (*b* in the figure).

When we forced cows to eat bracken in two consecutive periods with a 48-h pause inbetween, we obtained a bimodal excretion curve. Thus, cattle exposed sporadically to bracken feeding will yield pulses of

ptaquiloside in milk a few hours after starting to feed and for a period of more than 80 h after the animal has stopped eating bracken. This is a wide window for ptaquiloside to be present in milk. Despite the fact that cattle do not normally choose to eat bracken, they can be forced to eat it during periods of drought when bracken remains green, or when natural grasses are overgrazed, or when animals wander into dense bracken thickets. Therefore, there is a rather high risk to humans of ingesting milk containing ptaquiloside in areas where bracken is dominant. If a person drinks about 0.5 litres of milk daily from a cow producing 20 litres of milk per day, and this cow has eaten a subtoxic dose of ptaquiloside of 5,000 mg per day (as would be contained in 6 or 7 kg of fern, which typically might be eaten for a few days), this person will ingest 10.75 ± 1.45 mg ptaquiloside per day.

Although we cannot reach any definite conclusions at present about toxicity to humans at the intake levels reported here, as there are as yet no data on the toxicology of ptaquiloside in humans, the acute toxic effects of this chemical to rabbits at 50–100 mg per kg (ref. 9), as well as the expected consequences of ingesting a carcinogen and DNA-damaging compound at this concentration, strongly indicate cause for concern. It is certainly likely, in our view, that ptaquiloside in milk is responsible for the connection between bracken infestation and the incidence of gastric cancer in populations of farmers inhabiting cattle-range areas in Costa Rica¹⁰ and in other countries where bracken growth is dense (Andean South America, Central America and southern Mexico).

Miguel E. Alonso-Amelot

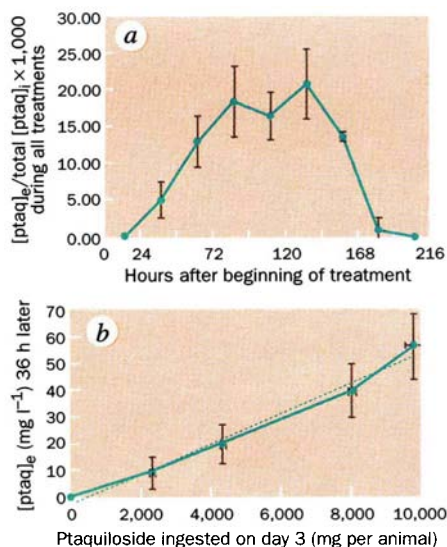
Uvidelio Castillo

Grupo de Química Ecológica,
Departamento de Química,
Facultad de Ciencias,
Universidad de Los Andes,
Mérida 5101, Venezuela

Barry L. Smith

Denis R. Lauren

New Zealand Pastoral Agricultural
Research Institute Ltd,
Ruakura Research Centre,
Hamilton, New Zealand



a, Ratio between ptaquiloside excreted through milk $[\text{ptaq}]_e$ and amount ingested $[\text{ptaq}]_i$ by cows ($n=6$) 38 h before, during 6 d of treatment with 6.0 kg fresh young bracken fronds containing an average of $4,422 \pm 79$ mg ptaquiloside. The approximate time required for ptaquiloside to appear in milk after the first ingestion of bracken fronds is 38 h. Error bars, s.d.. *b*, Variation of $[\text{ptaq}]_e$ (mg per 1 milk) by hour 86 in relation to the dose of $[\text{ptaq}]_i$ in the range 2,400–10,000 mg per animal per d, at a constant bracken frond intake of 6 kg per animal per d. Data for 5 animals showing the linear regression line (dotted). $[\text{ptaq}]_e$ (mg) = $-2.62(\pm 2.62) + 0.00562(\pm 4.3 \times 10^{-4}) \times [\text{ptaq}]_i$ (mg) ($r^2=0.9829$, $F=171.08$, $P=0.001$).

1. Niwa, H. et al. *Tetrahedron Lett.* **4**, 4117–4120 (1983).
2. Hirano, I. & Yamada, K. in *Naturally Occurring Carcinogens of Plant Origin* (ed. Hirano, I.) 87–120 (Kodansha Ltd & Elsevier, Tokyo, 1990).
3. Kushida, T. et al. *J. Am. Chem. Soc.* **116**, 479–486 (1994).
4. Evans, W. C. in *Bracken, Ecology, Land Use and Control Technology* (eds Smith, T. R. & Taylor, J. A.) 121–132 (Parthenon, Carnforth, UK, 1986).
5. Pamukcu, A. M., Erturk, E., Yalciner, S., Millil, U. & Bryan, G. T. *Cancer Res.* **38**, 1556–1560 (1978).
6. Evans, I. A., Jones, R. S. & Mainwaring Burton, R. *Nature* **237**, 107–108 (1972).
7. Alonso-Amelot, M. E., Pérez-Mena, M., Calcagno, M. P. & Jaimes, R. *Phytochem. Anal.* **3**, 160–164 (1992).
8. Alonso-Amelot, M. E., Castillo, U. & De Jongh, F. *Lait* **73**, 323–332 (1993).
9. Hirano, I. et al. *J. Vet. Med. Sci.* **55**, 979–983 (1993).
10. Villalobos, J. *Rev. Cost. Cienc. Med.* **6**, 131–135 (1985).

New window for spectroscopy

SIR—We have developed an imaging polarimeter system that can routinely record the Sun's spectrum with a precision of 10^{-5} in the degree of polarization. At this level, the whole solar spectrum is linearly polarized, even in the absence of any magnetic fields, due to coherent scattering processes in the solar atmosphere. The linearly polarized spectrum has a structural richness that is comparable to and often exceeds that of the ordinary, unpolarized

pixel sensitivities. As the polarimetric noise is then limited only by photon statistics, we achieve a polarimetric accuracy of 10^{-5} when ZIMPOL is used in combination with the solar telescope that has the largest photon-collecting area in the world, the McMath-Pierce facility of the National Solar Observatory (Kitt Peak, United States).

The second solar spectrum contains a wealth of unexpected spectral features

the solar atmosphere.

Quantum-mechanical interference between states with different J quantum numbers was first discovered in the solar spectrum for the H and K lines of ionized calcium at 3,965 and 3,933 Å (ref. 6). The Na I D_2 – D_1 lines illustrated here (b in the figure) provide a particularly clean example of this type of interference, while presenting us with new puzzles. Like in the double-slit experiment when each 'photon' has to pass both slits at the same time, each Na I scattering process has to go through both excited $J=3/2$ and $1/2$ states 'at the same time'.

The interference between the scattering amplitudes gives rise to sign reversals of the linear polarization around the D_1 line. Although the gross features of the overall polarization curve can be theoretically modelled, it has not yet been possible to explain the narrow polarization peaks at the two resonant frequencies. Although the narrow D_1 peak would require an atomic-physics explanation outside our current theoretical framework, the D_2 peak may have an explanation largely in terms of partial frequency redistribution coupled to polarized radiative transfer, although this needs to be worked out. In any case, the full explanation of the Na I D_1 – D_2 system is likely to lead to new insights in both atomic physics and radiative-transfer physics.

The Ba II 4,554-Å line (c in the figure) has contributions from various isotopes. The atomic levels of the odd isotopes are subject to hyperfine structure splitting, due to coupling between the electronic angular momentum and the nuclear spin. Our theoretical modelling shows that the polarization components in the blue- and red-line wings come from the hyperfine structure components of the odd isotopes, while the central component is due to the even isotopes. The theoretical fit is sensitive to isotope abundances.

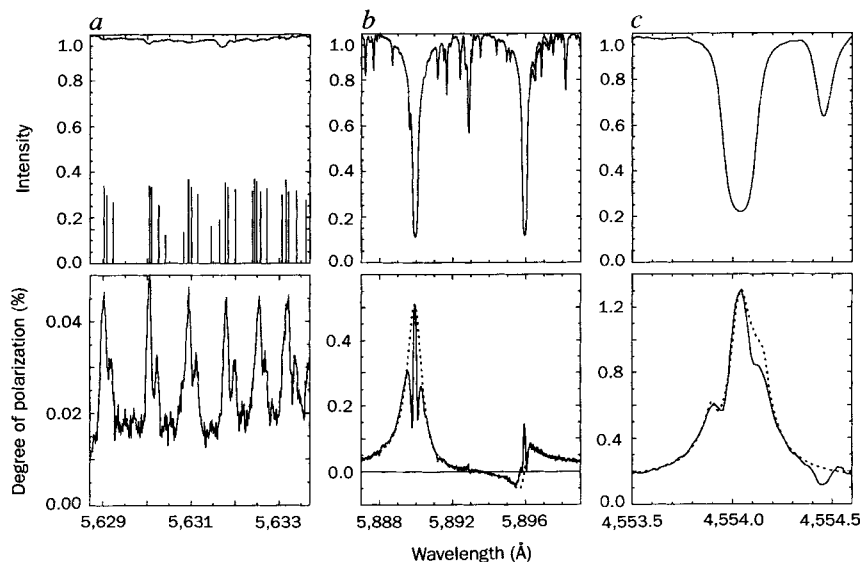
The second solar spectrum will be important in exploring the nature of solar magnetic fields via the Hanle effect. Much of the magnetic flux on the Sun is in a tangled, turbulent state, with mixed polarities within each spatial resolution element. Although such a field is invisible to ordinary Zeeman-effect polarimetry owing to cancellation effects within the resolution elements, such cancellations do not occur for Hanle depolarization effects in the second solar spectrum. The differential Hanle effect in combinations of spectral lines provides us with a new diagnostic window for the exploration of magnetoturbulence.

J. O. Stenflo

*Institute of Astronomy ETH Zentrum,
CH-8092 Zurich, Switzerland*

C. U. Keller

*National Solar Observatory,
PO Box 26732, Tucson,
Arizona 85726-6732, USA*



Examples of molecular scattering, quantum interference and hyperfine structure in the second solar spectrum (degree of linear polarization). a, C₂ lines; b, Na I D₂ and D₁; c, Ba II 4,554 Å. Recordings have been made with the spectrograph slit parallel to and 5 arcsec inside the north polar limb of the Sun. Positive polarization means that the electric vector is parallel to the nearest solar limb; negative polarization refers to the perpendicular direction. Bars in the left intensity diagram give the wavelengths and relative intensities of C₂ lines from laboratory data⁷. Theoretical, dotted curves in the middle and right polarization panels have been obtained from quantum-mechanical calculations with superposition of the contributions from the lines and continuum in proportion to their opacities.

spectrum. Not only is its appearance different, but it is shaped by quite different physical processes, as we illustrate below. We call it the "second solar spectrum", because it is as if the Sun has presented us with an entirely new spectrum to explore.

Our CCD (charge-coupled-device)-based polarimeter system, called ZIMPOL, uses piezoelastic modulation at 42- and 84-kHz rates and demodulates the signals within the CCD sensor by shifting the charges back and forth between the illuminated and hidden buffer image planes in synchrony with the fast modulation¹⁻⁵. This procedure eliminates the two main noise sources due to effects of atmospheric seeing and the variable CCD

that are signatures of different physical processes. Here we illustrate a few: molecular scattering, quantum interference, hyperfine structure and isotope effects. These effects are best studied close to the solar limb, where the polarization amplitudes are the largest because of the favourable scattering geometry.

Molecular lines like those of the C₂ molecule (a in the figure) are prominent in the polarized spectrum although they are almost invisible in the ordinary intensity spectrum. Because their behaviour critically depends on where in the Sun the molecules can form, the polarization amplitudes can be used as a new, sensitive temperature and pressure diagnostic for

1. Povel, H. P., Keller, C. U. & Stenflo, J. O. in *Solar Polarimetry* (ed. November, L. J.) 102-111 (NSO/SP Summer Workshop Ser. No. 11, Sunspot NM, 1991).
2. Keller, C. U. et al. Tech. Rep. No. 53 (LEST Found. Inst. Theoretical Astrophys., Univ. Oslo, 1992).
3. Stenflo, J. O., Keller, C. U. & Povel, H. P. Tech. Rep. No. 54 (LEST Found. Inst. Theoretical Astrophys., Univ. Oslo, 1992).

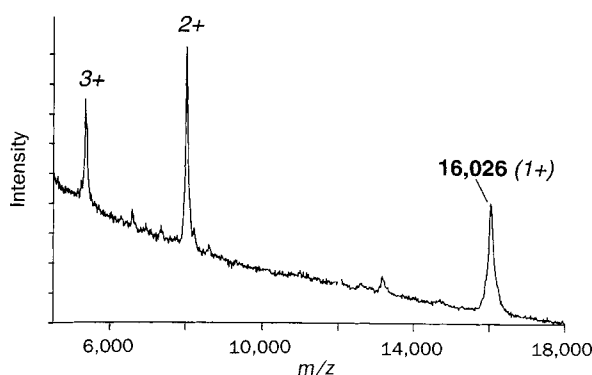
4. Povel, H. P. *Opt. Eng.* **34**, 1870-1878 (1995).
5. Stenflo, J. O. *Solar Magnetic Fields — Polarized Radiation Diagnostics* (Kluwer, Dordrecht, 1994).
6. Stenflo, J. O. *Astron. Astrophys.* **84**, 68-74 (1980).
7. Phillips, J. G. & Davis, S. P. *The Swan System of the C₂ Molecule* (Univ. California Press, Berkeley, 1968).

Human leptin characterization

SIR — Injections of leptin, the hormone encoded by the mouse *ob* gene, reduce body weight in wild type and *ob* mice¹⁻⁴. However, the weight-reducing effects of recombinant leptin expressed from bacteria in wild-type mice require relatively high doses of leptin¹⁻³. The basis for the high dosage requirement of recombinant leptin could be partly pharmacokinetic but could also be the result of post-translational modifications of the native protein.

To address this question we looked for differences in the molecular mass between recombinant and endogenous human leptin

from SDS-PAGE for mass spectrometric analysis. The method makes use of reversible, negative staining protocols (for example copper⁵ or zinc⁶ staining) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)⁷. We could readily see bands with as little as 3 pmol (50 ng) loading of refolded bacterial recombinant leptin on copper-stained (Bio-Rad) SDS gels measured by mass spectrometry. We excised protein bands from the gel, destained them, and extracted the protein into a MALDI matrix solution.⁸ Full details of this new protein extraction procedure will be given elsewhere.



Mass spectrum of endogenous human leptin. The MALDI mass spectrum is dominated by a single component (leptin) with a measured molecular mass of $16,026 \pm 9$. The three main peaks represent singly, doubly and triply protonated forms of the protein arising from the MALDI mass spectrometric process⁷. *m*, mass; *z*, charge.

tin. We isolated endogenous leptin using two stages of immunoaffinity chromatography from 50 ml serum pooled from three moderately obese human female donors (leptin concentration 54 ng ml^{-1}). We eluted the protein from a monoclonal antibody-affinity resin and subjected it to SDS-PAGE. The principal band, well separated from contaminating and irrelevant proteins, showed the same mobility as recombinant human leptin (apparent relative molecular mass 16,000); we estimated it to contain more than 250 ng protein.

Although the endogenous and recombinant human leptins have the same mobility after immunoblotting, SDS-PAGE has insufficient accuracy to exclude the possibility of subtle differences (for example, post-translational modifications). To measure accurately the molecular mass of the native protein, we developed a method to elute whole pro-

teins from SDS-PAGE for mass spectrometric analysis. The method makes use of reversible, negative staining protocols (for example copper⁵ or zinc⁶ staining) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)⁷. We could readily see bands with as little as 3 pmol (50 ng) loading of refolded bacterial recombinant leptin on copper-stained (Bio-Rad) SDS gels measured by mass spectrometry. We excised protein bands from the gel, destained them, and extracted the protein into a MALDI matrix solution.⁸ Full details of this new protein extraction procedure will be given elsewhere.

where (and are available directly from S.L.C and B.T.C.). Using this technique, we find the molecular mass of endogenous human leptin to be $16,026 \pm 9$ (see figure), a value identical, within experimental error, to the molecular mass calculated from the *ob* cDNA sequence minus the putative signal sequence, 16,024 (ref. 9). In a parallel experiment, recombinant leptin extracted from the gel gives a mass of $16,178 \pm 10$.

The presence of an amino-terminal start methionine in the recombinant leptin accounts for the mass difference observed between the endogenous and recombinant proteins.

We undertook further comparison of the proteins by performing in-gel trypsin and cyanogen bromide digests followed by extraction and MALDI-MS analysis. Except for the amino-terminal methionine on the recombinant protein, the fragmentation patterns of the recombinant and endogenous proteins are virtually identical. In addition, molecular mass measurements of the peptides generated after digestion indicate the presence of a carboxy-terminal disulphide bond in both the endogenous and refolded recombinant proteins (data not shown).

The results show that immunopurified leptin from human serum has the wild-type sequence with the removal of the predicted amino-terminal signal peptide and with the presence of a carboxy-terminal intramolecular disulphide bond. There is no evidence of other post-translational modifications. Further studies are required to determine the basis for the high dosage requirements of leptin to induce weight loss.

Steven L. Cohen*, **Jeffrey L. Halaas†‡**, **Jeffrey M. Friedman†‡**, **Brian T. Chait***

Laboratories of *Mass Spectrometry and Gaseous Ion Chemistry, and †Molecular Genetics and ‡Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue,

New York, New York 10021, USA

Larry Bennett, David Chang, Randy Hecht & Frank Collins

Amgen, Inc., 1840 DeHavilland Drive, Thousand Oaks, California 91320, USA

Correspondence to be addressed to B. T. C. E-mail: chait@rockvax.rockefeller.edu

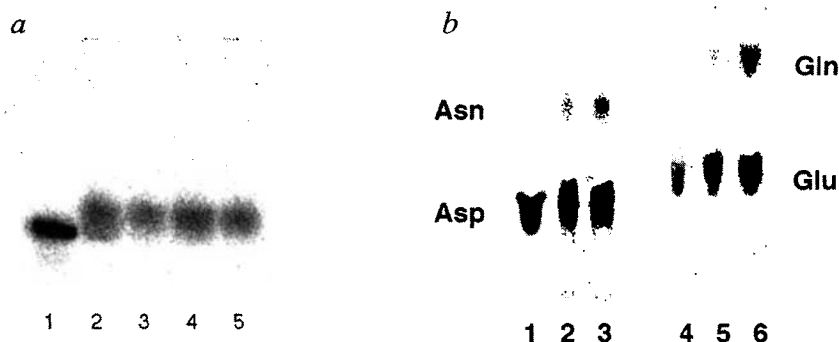
tRNA-dependent asparagine formation

SIR — It is commonly assumed that there are 20 aminoacyl-transfer (t)RNA synthetases, which form the different aminoacyl-tRNAs required for protein biosynthesis, in every organism. Direct acylation of tRNA with the amide amino acid, asparagine, is catalysed by asparaginyl-tRNA synthetase (AsnRS) in all organisms studied biochemically to date¹. Genomic analysis of eubacteria^{2,3} and a eukaryote⁴ also predict both cytoplasmic and organellar AsnRS activity. In Gram-negative eubacteria and in the cytoplasm of eukaryotic cells, glutaminyl-tRNA synthetase acylates tRNA^{Gln} directly with glutamine providing Gln-tRNA^{Gln}. However, in Gram-positive eubacteria, archaeobacteria and organelles Gln-tRNA^{Gln} formation is achieved by

transamidation of misacylated Glu-tRNA^{Gln} (refs 5–7). We now show that transamidation, and not direct acylation, provides Asn-tRNA^{Asn} for protein biosynthesis in the halophilic archaeobacterium *Haloferax volcanii*.

We investigated transamidation of mischarged tRNAs in *H. volcanii* as neither tRNA^{Asn} nor tRNA^{Gln} isolated from this archaeon could be aminoacylated *in vitro*⁸. *H. volcanii* total tRNA is considerably less well charged by Gln than by Glu in the presence of a dialysed S100 extract (data not shown). Comparable results were previously observed in *Lactobacillus bulgaricus*⁹, and are consistent with the existence of a tRNA-dependent transamidation pathway for the formation of Gln-tRNA^{Gln}. We also find that Asn is a signif-

1. Halaas, J. L. *et al.* *Science* **269**, 543–546 (1995).
2. Pelleymounter, M. A. *et al.* *Science* **269**, 540–543 (1995).
3. Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R. & Burn, P. *Science* **269**, 546–549 (1995).
4. Stephens, T. W. *et al.* *Nature* **377**, 530–532 (1995).
5. Lee, C., Levin, A. & Branton, D. *Anal. Biochem.* **166**, 308–312 (1987).
6. Ortiz, M. L., Calero, M., Patron, C. F., Castellanos, L. & Mendez, E. *FEBS Lett.* **296**, 300–304 (1992).
7. Chait, B. T. & Kent, S. B. H. *Science* **257**, 1885–1894 (1992).
8. Cohen, S. L. & Chait, B. T. *Anal. Chem.* **68**, 31–37 (1996).
9. Zhang, Y. *et al.* *Nature* **372**, 425–432 (1994).



Conversion of Asp-tRNA^{Asn} and Glu-tRNA^{Gln} by tRNA-dependent transamidation. *a*, Total tRNA from *H. volcanii*¹⁶ was aminoacylated and electrophoresed as described¹⁷ except that electrophoresis was only for 150 min to minimize deacylation. Lane 1, 2 μ M *Escherichia coli* tRNA^{Gln} + 5 nM *E. coli* GlnRS¹⁸; lanes 2–5, *H. volcanii* tRNA, *H. volcanii* S100 extract and [¹⁴C]Asp (lane 2), [¹⁴C]Asp + unlabelled Asn (lane 3), [¹⁴C]Glu (lane 4), [¹⁴C]Glu + unlabelled Gln (lane 5). *b*, Thin-layer chromatography of amino acids recovered from aminoacyl-tRNAs shown in *a* following alkaline deacylation. Lane 1, [¹⁴C]Asp; lane 2, [¹⁴C]Asp + tRNA; lane 3, [¹⁴C]Asp + tRNA + unlabelled Asn; lane 4, [¹⁴C]Glu; lane 5, [¹⁴C]Glu + tRNA; lane 6, [¹⁴C]Glu + tRNA + unlabelled Gln. Unless stated, reactions conditions were standard buffer¹⁶, 0.25 mg tRNA, 0.14 mg dialysed S100 extract, 20 μ M [¹⁴C]amino acid, 1.5 mM unlabelled amino acid.

icantly poorer substrate for tRNA aminoacylation than Asp in *H. volcanii*, suggesting the absence of an asparaginyl-tRNA synthetase activity (data not shown). This aminoacylation pattern contrasts with that observed with *L. bulgaricus*, which possesses conventional AsnRS activity¹. These results suggest that the formation of Asn-tRNA^{Asn} in *H. volcanii* proceeds via the transamidation of Asp-tRNA^{Asn} rather than by the direct charging of asparagine to tRNA.

We confirmed the formation of Asn-tRNA^{Asn} and Gln-tRNA^{Gln} by tRNA-dependent transamidation pathways by characterizing the products of aminoacylation reactions performed with radio-labelled amino acids (see figure). The identical migration pattern in each lane of *a* suggests that the tRNA is aminoacylated with the corresponding [¹⁴C]amino acid. In the absence of tRNA, we observed no conversion of Asp or Glu (*b*, lanes 1 and 4) indicating that the amidation process is tRNA-dependent. When [¹⁴C]Asp and [¹⁴C]Glu are incubated in the presence of *H. volcanii* total tRNA without amide donor, a small amount of [¹⁴C]Asp $\rightarrow$ [¹⁴C]Asn and [¹⁴C]Glu $\rightarrow$ [¹⁴C]Gln conversion takes

place, facilitated by traces of amide donors still present in the dialysed *H. volcanii* extract (*b*, lanes 2 and 5). However, in the presence of the corresponding unlabelled amino acid as amide donor, conversion of [¹⁴C]Asp-tRNA^{Asn} $\rightarrow$ [¹⁴C]Asn-tRNA^{Asn} and of [¹⁴C]Glu-tRNA^{Gln} $\rightarrow$ [¹⁴C]Gln-tRNA^{Gln} occurs (*b*, lanes 3 and 6). Thus, the correctly charged tRNA^{Asn} and tRNA^{Gln} species are formed via the mischarging/transamidation mechanism. Replacing Asn with Gln as amide donor gives 64% activity in [¹⁴C]Asp-tRNA^{Asn} $\rightarrow$ [¹⁴C]Asn-tRNA conversion, and replacing Gln with Asn gives 52% in [¹⁴C]Glu-tRNA^{Gln} $\rightarrow$ [¹⁴C]Gln-tRNA conversion; this suggests the presence of two separate transamidases.

Transamidation represents a significantly more complex route to aminoacyl-tRNA than conventional direct aminoacylation and requires enzymatic activities usually confined to intermediary metabolism of amino acids. This must result from either secondary adaptation to a hypersaline environment or the addition of Asn and Gln to the set of amino acids used for protein synthesis after the evolution of aminoacyl-tRNA syn-

thetases. The latter is unlikely, as the Archaea are believed to have evolved with Eucarya^{10,11} which contain a full complement of 20 cytoplasmic aminoacyl-tRNA synthetase genes. The requirement for transamidation may instead result from the inability of a single enzyme to discriminate between similar substrates (for example, Asp from Asn or Glu from Gln) sufficiently well to minimize translational errors arising from tRNA mischarging in a hypersaline environment (K. W. Hong and M. I., unpublished data). However, tRNA-dependent amino-acid transformations occur within the overall context of aminoacyl-tRNA, providing a larger, more diverse substrate for recognition. In a comparable fashion *Escherichia coli* valyl-tRNA synthetase discriminates mis-activated threonine after it has been attached to tRNA¹².

The tRNA-dependent transamidations described here, together with the various tRNA-dependent modifications of serine observed during selenocysteyl-tRNA synthesis¹³, define an important group of reactions not catalysed by aminoacyl-tRNA synthetases but essential for protein synthesis. This indicates that the aminoacyl-tRNA synthetases, although required, are not exclusively responsible for providing the aminoacyl-tRNAs necessary for messenger RNA translation as famously proposed in Crick's adaptor hypothesis¹⁴.

A common feature of these particular pathways for aminoacyl-tRNA synthesis is that a tRNA must first be mischarged with a non-cognate amino acid. This supports the proposal that any amino acid-codon chemical relationships which might have contributed to the origin of the genetic code would necessarily have been limited in their range¹⁵. Consequently, other aminoacyl-tRNAs might also be synthesized in the absence of a specific aminoacyl-tRNA synthetase in certain organisms such as archaeobacteria, raising the question of how many such enzymes are necessary to support accurate protein biosynthesis.

Alan W. Curnow*

Michael Ibba*

Dieter Söll

Department of Molecular Biophysics and Biochemistry,
Yale University,
New Haven, Connecticut 06520-8114,
USA

e-mail : soll@trna.chem.yale.edu

*Contributed equally to this work, so are listed alphabetically.

- Kim, S. I., Nalaskowska, M., Germond, J. E., Pridmore, D. & Söll, D. *Nucleic Acids. Res.* **24**, 2648–2651 (1996).
- Fleischmann, R. D. et al. *Science* **269**, 496–512 (1995).
- Fraser, C. M. et al. *Science* **270**, 397–403 (1995).
- <http://genome-www.stanford.edu/Saccharomyces/>
- Schön, A., Kannangara, C. G., Gough, S. & Söll, D. *Nature* **331**, 187–190 (1988).
- Wilcox, M. & Nirenberg, M. *Proc. Natl. Acad. Sci. USA* **61**, 229–236 (1968).
- White, B. N. & Bayley, S. T. *Can. J. Biochem.* **50**, 600–609 (1972).
- Gupta, R. J. *Biol. Chem.* **259**, 9461–9451 (1984).
- Schön, A., Hottinger, H. & Söll, D. *Biochimie* **70**, 391–394 (1988).
- Doolittle, R. F. *Proc. Natl. Acad. Sci. USA* **92**, 2421–2423 (1995).

- Brown, J. R. & Doolittle, W. F. *Proc. Natl. Acad. Sci. USA* **92**, 2441–2445 (1995).
- Fersht, A. R. & Kaethner, M. *Biochemistry* **15**, 3342–3346 (1976).
- Baron, C., & Böck, A. in *tRNA: Structure, Biosynthesis and Function* (eds Söll, D. & RajBhandary, U. L.) 529–544 (ASM Press, Washington, DC, 1995).
- Crick, F. H. C. *Symp. Soc. Exp. Biol.* **12**, 138–163 (1958).
- Jimenez-Sanchez, A. J. *Mol. Evol.* **41**, 712–716 (1995).
- Bayley, S. T. in *Protein Biosynthesis in Bacterial Systems, Methods in Molecular Biology* vol. 1 (eds Last, J. A., & Laskin, A. I.) 89–110 (Decker, New York, 1971).
- Varshney, U., Lee, C.-P. & RajBhandary, U. L. *J. Biol. Chem.* **266**, 24712–24718 (1991).
- Hong, K. W. et al. *EMBO J.* **15**, 1983–1991 (1996).

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

Crossing the threshold

Frank González-Crussi

The Body's Edge: Our Cultural Obsession with Skin. By Marc Lappé. Holt: 1996. Pp. 242. \$22.50.

THE skin is much more than a simple boundary of the physical self, a simple frontier where the person ends and the outer environment begins. It is more than a mere enclosure that restrains our viscid interior from oozing out, thus guarding our integrity. It is also a custom-house for multifarious incident influences, a sensitive radar alert to the subtlest perturbations of the ambience, a clearing-house for impulses generated within the body or without, and a protective barrier, a living screen of unmatched efficiency, whose discernment fences in the good and fences out the untoward. Nor is this all: criss-crossed by a dense, intricate tuft of nerves and sensory receptors, the skin is the focal point of aesthesia, the conscious faculty that contributes to physical preservation, and, just as aptly say the ascetical foes of sensuality, to our spiritual perdition and moral ruin.

So complex and fundamental a part of the body could not fail to become the subject of intense scientific investigation; and it is rather surprising that its rich evocativeness has not made it more often the central topic of literary dissertation. In *The Body's Edge*, Marc Lappé approaches the skin from the plural viewpoint of the scientist, the sociologist and the cultural anthropologist — and at times, though (perhaps fortunately) not often, the surrogate poet. By his own avowal in the preface, he “wanted to go beyond the simple biology of the skin to explore how we define our ‘edge’ or boundary with the outer world”.

This professed aim is ambitious indeed. Its thorough development would require considerably more space than the relatively modest one comprised in a little over two hundred pages. It could be, God guard us, the work of a lifetime. Yet the author gallantly charges forward, armed with his record of past accomplishment (five previous books, including well-turned-out works of scientific popularization), his abiding interest in toxicology and its social implications and his undaunted optimism — a quality, this last one, without which no one could pos-

sibly feel impelled to write such a book.

Consider the scope of the work. Traditional dermatology is disposed of in one chapter; dermatological manifestations of systemic diseases in another. Cosmetology is dealt with in twenty pages. But the history of cosmetology, as acknowledged by the author in the final chapter,



At full stretch: James Morris, “elastic skin man” (c. 1890).

is “a long and broken trail of vanity, illusion and medical catastrophes”. Quite so, and therefore chroniclers of human folly were ever burdened with a Sisyphean, overwhelming task. An instance of just such human shortcoming is dealt with in a separate chapter, entitled “The Silicone Story”. It is a history of the US government-approved injection or surgical implantation of industrial silicone products as a means to reshape bodily parts perceived as unattractive and to smoothen wrinkled, aged skin. The author, who directs a centre for ethics and toxic substances, gives us a well-informed perspective of this episode, sadly brimful with human vanity and not

exempt from medical catastrophes.

Cosmetology, of course, leads us to ponder the broader question of the human preoccupation with bodily decoration, including tattooing, and from here it is an easy transition to evolutionary speculation. Other species have developed bright accoutrements, flamboyant plumage or polychrome *derrière* to attract potential mates. If human beings can flaunt no comparable endowment, the author tells us with somewhat facile discursive fluidity, it must be because their use of pigments, tattoos, lipstick, eyeshadow and assorted devices for enhancement or concealment has barred them from the natural process of beautification that sexual selection promotes in the wild. This claim may smack of the exaggerating brand of speciosity, but it was surely made in the same spirit as the assertion, in an earlier chapter, that ancient Chinese medicine anticipated modern knowledge of the immunological function of the skin by nearly two thousand years.

The Body's Edge is a thoughtful, up-to-date, highly readable work, likely to hold the interest of the nonmedical public. Dermatologists and allied experts will probably find many of the chapters uninformative or superficial, particularly those devoted to anatomy, function and diseases of the skin. But even these readers would benefit from tracing the “long trail of vanity, illusion and medical catastrophe” that is historically annexed to their field, and pondering the many social and other varied factors, of apparently no direct medical purport, that influence the health of the skin. Thus the reader is made to see how environmental depredation, by thinning the ozone layer, permits cancer-promoting rays to filter through and wreak their cutaneous havoc. Similarly, cultural attitudes, through their obsessive insistence in maintaining a youthful appearance, promote bizarre, misguided practices no less damaging to the skin. The idea of a subtle connectedness of all things in the Universe, so plain to the ancients and so deliberately ignored by modern specialists, acquires new relevancy in thoughtful, well-crafted works. *The Body's Edge* is one of these. □

Frank González-Crussi is in the Department of Pathology, Children's Memorial Hospital, 2300 Children's Plaza, Chicago, Illinois 60614, USA.

Unto the sweet bird's throat

Peter Marler

Bird Song: Biological Themes and Variations. By C. K. Catchpole and P. J. B. Slater. Cambridge University Press: 1995. Pp. 248. £19.95, \$32.95.

WHETHER it be the crow of the cock or the music of nightingales, almost all birds have something that can broad-mindedly be called a song. Most remarkably, nearly half of all the 9,000 known bird species have to learn to sing. Without the opportunity to learn, they develop a song unlike any to be heard in nature.

But the raw numbers are deceptive. Most of the 4,000 or so probable song-learners are perching birds, coming from just one of the 27 orders into which taxonomists classify the living birds of the world, thus excluding all marine and freshwater birds, waders, galliforms, doves, hawks and owls. Despite their near-plurality, the learners are an exclusive group, restricted to parrots, hummingbirds and above all the prolific suborder of the Passeriformes known as the oscines or songbirds, characterized especially by their complex vocal apparatus — a first clue to their supremacy as songsters. But to learn to sing, a bird also needs a brain that is up to the task of identifying and memorizing the

What unusual selection pressures have led to the cultural transmission of song patterns from generation to generation, much like our own speech behaviour? In *Bird Song*, two distinguished researchers, Clive Catchpole and Peter Slater, review this and other issues, paying special attention to the singularities of songbirds. The thoughtful discussions of sound production and perception, and problems of physical analysis and description, are most welcome, given the complexity and variability of many birdsongs. A cautionary account of the difficulties encountered in studying geographical variation, and its functional significance, gives rise to some scepticism about the continuing value of the concept of local dialects. The curious blend of species-specificity and extreme variability that enriches the communicative potential of birdsongs also complicates the task of the experimenter striving for representative samples of songs for tutoring and playback.

The accounts of song-learning review some of the striking species differences in when, how and which songs are learned. In harmony with recent trends, functions of song are dwelt on at length, including relationships of sound transmission to the environment and time of day, the role of male song in territoriality and in attracting and stimulating females, and the part played by female preferences in the evolution of song. A comprehensive bibliography of some 700 entries adds to the value of the book as a scholarly resource.

If there are problems, they reflect the state of the field. Singing behaviour has many different functions, and the emphasis varies from species to species. Again and again, the authors conclude an analysis of a positive result with negative findings on other species. It is salutary to be reminded of such contradictions, especially given the inclination of some researchers to ignore those that are theoretically inconvenient; but a failure to comment further sometimes leaves the reader uncertain whether the intention is to reduce credibility or simply limit generality. More counsel from the authors on how next to proceed would have been useful, serving to lighten their cautious and generally retrospective viewpoint.

We are left with a healthily quantitative appreciation of the two main roles of bird-song, mate attraction and territorial defence, but conceptually we have not made much progress beyond such past authors as R. D. Howard and N. Tinbergen. It remains unclear what special functional requirements are served by learned song. What kind of creative impulse drives some species to develop largely inventive, sometimes borrowed repertoires of a hundred or more songs, whereas a single slavishly imitated song suffices for a white-crowned sparrow? Species differences in female preference and susceptibility to

physiological stimulation, and perhaps an appreciation for vocal invention, are all prime issues here. Might not brain mechanisms for inventiveness turn out to be as interesting as those for imitation? Many mysteries remain, biological, ecological and psychological, which is what makes the singing behaviour of birds so fascinating. □

Peter Marler is in the Section of Neurobiology, Physiology and Behavior, University of California, Davis, California 95616, USA.

■ Also just published is *Ecology and Evolution of Acoustic Communication in Birds* edited by D. E. Kroodsma and E. H. Miller. Comstock/Cornell University Press, \$75 (hbk), \$35 (pbk).

Chattering classes

Derek Bickerton

The Evolution of Communication. By Marc D. Hauser. MIT Press: 1996. Pp. 760. \$55, £44.95.

FEW writers have so far even attempted a general overview of animal communication, so Marc Hauser's book is timely if not overdue. Hauser brings to the task a formidable knowledge of the field (his bibliography contains some 1,500 items) plus a lucid style and an infectious enthusiasm that carry one smoothly through an immense maze of information and make complex biological theories accessible even to the uninitiated. For anyone concerned with the comparative study of communication, this book is likely to remain an indispensable source for some time to come.

A caveat, however, is in order. That Darwin leaned heavily on the comparative method does not automatically make every comparative study evolutionary. Hauser claims to have adopted a fourfold Tinbergian framework, analysing signals in terms of their mechanical infrastructure and functional significance as well as their ontogenetic and phylogenetic development. As expected, there is a full chapter devoted to each of the first three topics, but none to the fourth, the most crucial from an evolutionary perspective, and references to phylogeny are fleeting and tangential throughout. Hauser is more concerned with currently hot issues such as the relative adaptive value of honest versus dishonest signals than with the ancestry of either kind.

What might one legitimately expect from a book about the evolution of communication? Take mating signals, one of the three broad classes (the others are 'survival' and 'social' signals) into which Hauser divides his material. Clearly, as soon as sexual reproduction began, it

IMAGE
UNAMIGABLE
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FOR REASON
REASONS

Thomas Mangelsen/BBC Natural History Unit

Supreme songster: the western meadowlark (*Sturnella neglecta*).

sounds of others and guiding the production of imitations. In fact, oscine birds are all but unique in having distinctive brain mechanisms expressly designed for this task. The caveat is needed because parrots and perhaps hummingbirds have independently achieved the same end with equivalent but neuroanatomically distinct circuitry.

became adaptive for organisms to distinguish opposite-sex conspecifics from same-sex conspecifics, heterospecifics, algae, bits of rock and so on. But how exactly did anything one could call 'communication' evolve from pre-existing and purely fortuitous marks of distinction? We don't learn this because — apart from a few pages on warning colours in insects — Hauser tackles no creatures less complex than frogs.

Indeed the jacket-blurb's claim that the book "covers an extraordinarily diverse group of organisms" is wildly inflated. Most of the text concerns only frogs, birds and primates; there are only the briefest of passing references to marine mammals, fish, canids or any brand of herbivore, let alone invertebrates. Yet the book contains a 30-page section on echolocation in bats. Hauser's explanation for this also explains the book's heavy bias in favour of acoustic systems; that the mechanisms underlying bat echolocation may provide insight into truly communicative systems, "including human speech".

Herein lies the gravest flaw in the book. Instead of tackling his topic head-on, and letting the chips fall where they may, Hauser seeks throughout to integrate human language into the overall animal pattern. He takes it as gospel that "language must be viewed as a communicative form that evolved from earlier forms". But more than two decades ago the psychologist Richard Gregory suggested an alternative explanation, equally valid in terms of evolution, yet much more plausible — that language resulted from the co-option of some pre-existing, non-communicative faculty for a communicative function. Hauser fails even to mention this possibility.

Although Hauser presents fair and accurate summaries of what linguists have had to say about language evolution, he strikingly fails to appreciate the problems concerning the evolution of syntax that some of these linguists have raised, and goes instead for the easy bits — he writes far more about babbling than about syntax, and his account of language ontogeny peters out when it reaches the one-word stage. Yet, as attempts to teach language to other species have consistently shown, syntax most sharply distinguishes humans from nonhumans, and therefore stands in most need of evolutionary explanation.

To Hauser, however, the core of language is meaning rather than syntax. Unfortunately, he fails to appreciate that a necessary prerequisite is not necessarily an ancestral trait. So one can concede vervet monkeys' ability to distinguish pythons from martial eagles, their ability to label this distinction vocally and the fact that no creature lacking these abilities could achieve language, without conceding that vervet alarm calls are directly

ancestral to words. Words characteristically refer in the absence of their referents; vervet calls either refer in their referents' presence (when eagles or pythons appear) or don't refer at all (when used deceptively). Hauser's discussion of reference ignores this crucial distinction.

The study of language evolution will not advance until linguists and biologists treat each others' concerns with as much respect as they treat their own. Sadly, Hauser has not followed this course, and I confidently predict that his proposed search for communicational antecedents of language will prove fruitless. But he is by no means an unsceptical continuist, and some of the best things in the book are the experiments he suggests for delving more deeply into what vervet calls really mean. Let's hope he gets to do them while learning the Gregorian chant entitled "Co-option". □

Derek Bickerton is in the Department of Linguistics, University of Hawaii at Manoa, Moore Hall 569, 1890 East-West Road, Honolulu, Hawaii 96822, USA.

Think again

Igor Aleksander

After Thought: The Computer Challenge to Human Intelligence. By James Bailey. *BasicBooks*: 1996. Pp. 277. \$25.

DESPITE the ubiquity of digital computers and their ever-improving prowess in controlling space missions or bringing the information superhighway into our homes, it may be fairly hard to excite the reader of popular science with a tale of digital wonders. So ten out of ten to James Bailey for trying. The publishers describe his book as an indication that "the real computer age is just beginning" and that parallel computers will, at last, surpass the thinking abilities of humans, this time merely by evolving so to do.

Put simply, Bailey (a former manager of the US parallel-computer company Thinking Machines Inc.) is writing about the surprising and unexplored 'adaptive' or 'emergent' properties of systems that consist of many simple processors interacting with one another. The challenge to human intelligence is that because scientific culture and the thought patterns of human beings are serial, these emergent properties are hard to understand and even harder to use. The good news is that the giant machines might not need people who think serially to program them — they will work it all out for themselves. Emergence is, indeed, the key issue: throw thousands of microprocessor chips

together, allow them to communicate, and sensible, albeit incomprehensible, computing power emerges.

Putting aside the plausibility of this tale, Bailey has set out to make this potentially over-technical story more palatable by turning it into a perspective on thinking processes in the history of science. One of his heroes is Kepler, whose laws, he suggests, emerged in a somewhat serendipitous fashion, through sheer perseverance, lateral thinking and general playing with Tycho Brahe's astronomical data collected over 20 years. Indeed, the author drops a sufficient number of names of impressive thinkers, from Aristotle through Nietzsche to Danny Hillis (the designer of Thinking Machines' Connection Machine), to make the book an attractive competitor in the popular-science market.

Personally, though, while I think that emergence within interactive processes is one of the most fascinating questions of our age, I find the recurring reference to not only a computing revolution but also a revolution in scientific philosophy somewhat irritating. It might also be misleading for a general readership. There is no doubt that Bailey is right in saying that emergent properties are not well understood. Where he may be wrong is in turning this ignorance into a virtue. His argument centres on the fact that classical artificial intelligence, based as it is in logic, that is, formalized (dreadfully serial) human thought, has not produced great successes. So the adaptive and emergent systems based on unconstrained machine 'thought' will put this right. He has coined the word "intermath" which he claims to be the basis of topics such as neural networks, genetic algorithms, cellular automata and artificial life. The characteristic of 'intermath' is that it will drive the computers of the future without being understood by the humans of the future. For those of us who are trying (and, on odd occasions, succeeding) to provide a mathematical underpinning for these topics, this seems to advocate a rebellion against doing some interesting work just because it turns out to be hard.

Emergence in assemblies of simple computing nodes is fascinating, particularly because it does yield to classical analysis. This is why the papers on neural nets by John Hopfield in the early 1980s brought the subject back into the limelight after years in the dark. He equated the progress of computation in such a net to that of a simple equilibrium-seeking energetic system such as a ball falling into a trough. Another example is a discovery first described by Stuart Kauffman in the late 1960s that interacting genes simulated as very simple digital elements with random functions have an astonishing degree of emergent stability.

He expressed this as an artificial epigenesis and differentiation and found it to be surprisingly similar to that in living organisms. Bailey refers to this work and its current solution (which is based on the low probability of information transmission in the gene model) but fails to see that this is not some fancy 'intermath'. It is circuit design of a classical kind that can be understood by first-year engineering students armed with a bit of boring old probability theory.

One should also bear in mind that emergence is well-trodden ground. The computer-design pioneer and multimath John Von Neumann worked with cellular automata with emergent properties (such as self-replication) in the early 1950. And the mathematician John Conway published his celebrated "game of life" before 1970. The game also relies on the emergent properties of a two-dimensional network of nodes with simple rules, as Bailey fully acknowledges. The point is that emergence has been accepted and studied as an interesting phenomenon and has not in fact been rejected because of an occasional lack of mathematical tractability, which is what Bailey implies.

Another theme in the book is that only now have computers become parallel enough for emergence to be properly applied. But even the Connection Machine, which Bailey sees as an example of an engine that will lead to the new era, has been around for more than ten years, and not much of an 'intermath' revolution is in evidence — just some interesting applications that are mentioned in the book. Of course, the author feels that evangelists such as himself are needed to bring the revolution about, the message being the seeming heresy of letting the computer get on with it without attempting to impose the limitations of human thought on its programs.

Putting the excesses of this view aside, the book has compelling and interesting qualities. It is written as an inventive structure dividing scientific history into concern with 'place and geometry' in antiquity, 'pace and industry' from the Middle Ages on, and 'pattern' as the mark of science that should guide us towards the next millennium. But perhaps its strongest commendable quality is that it might attract readers of popular science, particularly younger ones, to the fascinating topic of emergent and adaptive computation. This may provide a more adventurous reason for becoming a computer scientist or engineer than the prospect of making systems for endless browsing on the Internet. □

Igor Aleksander is in the Department of Electrical and Electronic Engineering, Imperial College, Exhibition Road, London SW7 2BT, UK.

How much is an elephant worth?

Thomas E. Lovejoy

The Value of Life: Biological Diversity and Human Society. By Stephen R. Kellert. *Island/Shearwater*: 1996. Pp. 263. \$24.95.

The Sixth Extinction. By Richard Leakey and Roger Lewin. *Doubleday/Weidenfeld and Nicolson*: 1995. Pp. 271. \$24.95, £18.99.

Conservation and Biodiversity. By Andrew P. Dobson. *W. H. Freeman/Scientific American Library*: 1996. Pp. 264. \$32.95, £19.95.

THE major biological transformation of the planet that is already well under way can be viewed from a variety of perspectives. *Conservation and Biodiversity* is essentially a biologist's analysis of the challenges and solutions. *The Sixth Extinction* sets the biodiversity crisis in the context of the history of life. And *The Value of Life* examines human attitudes as the ultimate causes of the problem and means of redress.

In *The Value of Life*, Stephen Kellert contemplates the various values humans accord to other living things. There is much here that is new scholarship, at least in book form, and in that sense the volume is far the richest of the three for the conservation professional. The author identifies nine basic values, from utilitarian to moralistic. He also adds a tenth, theistic, in a brief consideration of the Kalahari Bushmen. Missing (in my view) is the 'library' value, the inestimable amount we can learn from studying the products of evolution, and which can strengthen the utilitarian argument.

The Value of Life is immensely stimulating, tending to inspire new questions with every one it answers. The excellent graphics help a great deal in the interpretation. Most of the work focuses on the attitudes of Americans. Some of the findings are not that surprising, for example that urban dwellers — in a biodiversity echo of Rousseau — tend to relate more romantically to wildlife than rural dwellers, especially when it comes to questions about, say, wolf reintroduction. In a survey of people's attitudes to groups of organisms, birds come out top, invertebrates bottom. Whales have emerged over the past couple of decades with a special status owing to our increased awareness of their intelligence and songs. This effect is not yet pronounced in Norway and Japan, where harvest of these mammals is still very much part of the national culture. Education has a strong effect on humanistic and moralistic values, but the decline in the latter among older Americans could be as much generational as a real reflection of ageing.

The comparison of the United States, Japan, Germany and Botswana is intriguing, with Japan having a much stronger controlling attitude to nature. The saddest note in the book is the decay among Bushmen of feelings of intimacy and kinship with nature, caused by cash incentives for harvest. Analysis of specific situations shows that attitudes vary greatly depending on the kind of animal and its natural history. The last two chapters usefully consider how knowledge of this sort should inform wildlife management, bureaucracies and education.

Conservation and Biodiversity displays the high-quality text and illustrations that one has come to expect of books in the Scientific American Library series, although, amazingly, Evelyn Hutchinson's, Stuart Pimm's and Timothy Wirth's surnames, and Costa Rica's INBio (National Institute for Biodiversity), are misspelled. The coverage is comprehensive, ranging from the ample chapter on "What is Biodiversity?" through to causes of extinction, including extensive sections on habitat fragmentation and loss, and *ex situ* and *in situ* conservation. "Biodiversity in a Changing World" gives excellent coverage from human demographics to climate change, and "The Wealth of Nature" deals with topics as diverse as bio-prospecting and ecotourism. Although direct citations do not appear in the text, the book contains a useful bibliography, making it eminently suitable for a course on the subject.

The Sixth Extinction, another superb work from the partnership of Richard Leakey and Roger Lewin, provides a well-illustrated tour of the history of life on Earth and describes Leakey's own personal odyssey, from his study of early hominids to his becoming a concerned conservation protagonist. It is a compelling introduction, grounded in the best modern science (from chaos theory to hominid evolution), to the reasons why the sixth extinction is generically different from previous mass extinctions — and certainly not in humanity's interest. Regrettably, there is no mention of the implications of human-induced climate change, but this does not detract from the book's value to the lay person.

The authors' treatments of the African elephant population and its management exemplify the different approaches of the three books. Dobson considers the issue in terms of the most effective culling strategy, whereas Kellert looks at it in terms of the value-sets of the pro- and anti-culling forces. Leakey, on the other hand, makes a compelling case that elephants, like whales, are special animals, so ethics should rule out any use. One must conclude that any interested person should seek a variety of perspectives. □

Thomas E. Lovejoy is at the Smithsonian Institution, 1,000 Jefferson Drive, SW, Suite 320, Washington DC 20560, USA.

Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer

Tewis Bouwmeester, Sung-Hyun Kim, Yoshiki Sasai, Bin Lu & Eddy M. De Robertis

Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, California 90095-1737, USA

An abundant cDNA enriched in Spemann's organizer, *cerberus*, was isolated by differential screening. It encodes a secreted protein that is expressed in the anterior endomesoderm. Microinjection of *cerberus* mRNA into *Xenopus* embryos induces ectopic heads, and duplicated hearts and livers. The results suggest a role for a molecule expressed in the anterior endoderm in the induction of head structures in the vertebrate embryo.

The organizer experiment¹ demonstrated that the dorsal lip of the amphibian gastrula has, when transplanted, the ability to recruit or organize neighbouring cells to form a twinned body axis. The grafted tissue has potent inducing activities, leading to the formation of a secondary central nervous system, dorsal mesodermal tissues, such as somites, and a secondary gut. Interest in the multiple activities of the organizer has led to the isolation of many genes expressed specifically in the dorsal lip of the *Xenopus* gastrula. Their products include nuclear proteins, mostly homeo-domain proteins, such as goosecoid², Xlim-1 (ref. 3), XFKH1/pintallavis^{4,5}, Xnot⁶, Xnot-2 (ref. 7), Siamois⁸, Otx2 (refs 9, 10) and Xanf-1 (ref. 11), and secreted proteins such as noggin¹², follistatin¹³, chordin^{14,15} and members of the transforming growth factor (TGF)- β family of growth factors¹⁶⁻¹⁸. *In situ* hybridization studies suggest that the organizer consists of multiple overlapping domains defined by these gene markers^{16,19}, and the task of mutating each of the mouse homologues of these organizer-specific genes, to analyse how they interact with each other, is under way²⁰.

The organizer region leads not only to the induction of multiple tissue types, but also to the development of anteroposterior polarity. Spemann recognized that early dorsal lips could induce entire axes, including head structures, but older dorsal lips would lose the ability to induce head, leading only to the formation of trunk-tail structures²¹. Studies in the mouse have provided genetic evidence for the existence of a head organizer region. Inactivation of *Lim-1* leads to mice lacking all head structures anterior to rhombomere 3, but having normal trunk-tail structures²². Mutations of murine *Otx2* and, to some extent, *HNF3 β* also cause severe deletions anterior to rhombomere 3 (refs 20, 23). In *Xenopus*, several experimental treatments can lead to the formation of normal trunk-tail structures lacking structures anterior to the hindbrain²⁴⁻²⁶. It seems that the formation of the anterior head region, where *Hox* genes are not expressed, is regulated differently from the hindbrain and trunk-tail region.

To explore further the molecular complexity of Spemann's organizer, we performed a differential screen for dorsal-specific cDNAs. The second most abundant mRNA found in the dorsal region of the *Xenopus* gastrula encodes a novel putative secreted protein designated cerberus. The *cerberus* mRNA has potent inducing activity in *Xenopus* embryos, leading to the formation of ectopic heads. Unlike other organizer-specific factors¹²⁻¹⁷, *cerberus* does not modify the fate of mesoderm, but instead suppresses the formation of trunk-tail mesoderm. It defines an anterior dorsolateral domain of the gastrula, including the leading

edge of the deep layer of the dorsal lip, a region that, as shown here, gives rise to foregut and midgut endoderm. It also promotes the formation of cement gland, olfactory placodes, cyclopic eyes, forebrain and duplicated heart and liver (a foregut derivative). The expression pattern and inducing activities of *cerberus* suggest an important role for a factor expressed in prospective foregut endoderm in the induction of the head region of the embryo.

A putative secreted protein encoded by *cerberus*

A differential screening approach was used to identify abundant dorsal-specific cDNAs without bias as to their function. As shown in Table 1, five previously known and five new cDNAs were isolated. The most abundant dorsal-specific cDNA was *chordin* (*chd*), with 70 independent isolates; the second most abundant cDNA was isolated 11 times, and was named *cerberus* (after a mythological guardian dog with multiple heads). The *cerberus* cDNA encodes a polypeptide of 270 amino acids, with an amino-terminal hydrophobic signal sequence and a carboxy-terminal cysteine-rich region (Fig. 1a). Preliminary experiments indicate that the cerberus protein is secreted into the culture medium of mRNA-injected *Xenopus* oocytes (T.B., unpublished observations). The amino-acid sequence of cerberus and the spacing of its nine cysteine residues were not significantly similar to other proteins in the databases (NCBI-Gen Bank release 93.0). We conclude that *cerberus* is an abundant dorsal-specific cDNA that encodes a novel secreted protein.

Demarcation of an anterior organizer domain

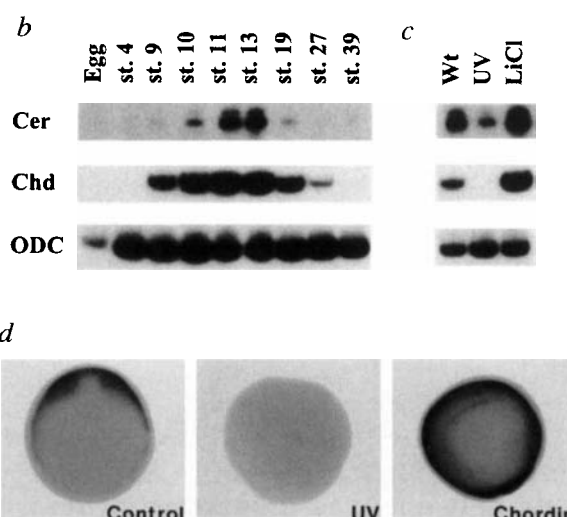
The *cerberus* mRNA is expressed at low levels in the unfertilized egg, and zygotic transcripts increase at stage 10 (Fig. 1b). Expression continues during gastrula and early neurula, rapidly declining during neurulation. Importantly, zygotic transcripts of *cerberus* accumulate about one hour later than those of *chd*, suggesting that *cerberus* could act downstream of the *chd* signal (Fig. 1b).

Whole-mount *in situ* hybridizations²⁷ show that *cerberus* zygotic expression becomes detectable in the yolk endomesodermal cells located in the deep layer of the organizer, in a domain that includes the leading edge of the most anterior cells and extends into the dorsal half of the floor of the blastocoel at stage 10 $\frac{1}{2}$ (Fig. 2a-c). In sections, *cerberus* mRNA is found in the deep yolk cells (Fig. 2b), which by stage 10 $\frac{1}{2}$ are separated from the neuroectoderm by a virtual space, called Albert Brachet's cleft (Fig. 2b, k), which in *Xenopus* forms long before the bottle cells of the external blastopore lip start ingressing²⁸⁻³⁰. Expression of *cerberus*

a

MLNLVLRICI	IVCLVNDGAG	KHSEGRERTK	TYSLNSRGYF	40
RKERGARRSK	ILLVNTKGLD	EPHGHGDFG	LVAEFLDSTR	80
THTRKEPDM	NKVLFSTVA	HGNKSARRKA	YNGSRRNIFS	120
RRSFDKRNT	VTEKPGAKMF	WNNFLVKMNG	APQNTSHGSK	160
AQEIMKEACK	TLPTQNIHV	ENCDRMVIQN	NLCFGKICISL	200
HVPNQDQRN	TCSHCLPSKF	TLNHLTLNCT	GSKNVVKVVM	240
MVEECTCEAH	KSNFHQTAQF	NMDTSTTLHH		270

FIG. 1 *Xenopus cerberus* encodes a putative secreted protein transiently expressed during embryogenesis. **a**, Deduced amino-acid sequence of *Xenopus cerberus* protein. The signal peptide sequence and the 9 cysteine residues in the C terminus are in bold. Potential N-linked glycosylation sites are underlined. In a BLAST database search, *cerberus* showed limited similarity only to the mammalian Dan protein, a possible tumour suppressor proposed to be a DNA-binding protein⁴⁷. However, the spacing of the cysteine residues and a crucial histidine in the proposed Dan zinc-finger domain are not conserved, indicating that the weak conservations identified by the computer might not be significant. **b**, Temporal expression of *cerberus*. Total RNA, isolated from the indicated stage (st.) of development, was analysed by RT-PCR for levels of expression of *cerberus* (Cer), *chordin* (Chd) and *ornithine decarboxylase* (ODC), which serves as a loading control. Zygotic expression of *cerberus* starts 1 h after *chd* activation, and is transiently expressed during gastrulation and neurulation. Expression of *cerberus* can be observed in unfertilized egg and blastula stages at low levels in longer exposures. **c**, RNAs from control (Wt), ultraviolet (UV)- or LiCl-treated embryos analysed by RT-PCR at stage 11. The relative intensity differences with **b** are due to differences in exposure time. **d**, *In situ* hybridization of wild-type controls, UV-treated and *chordin*-injected stage-11 embryos hybridized with *cer* antisense RNA.



METHODS. RNA was isolated from the indicated stages of development with the RNA STAT kit (Tel-Test). Quantitative RT-PCR and primers were as ref. 15, except: *cer* (forward primer (F), 5'-GCTGAACATTTGATCCACC; reverse primer (R), 5'-ATGGCTTGATTCCTGTGGGGC; 28 cycles); *chd* (F, 5'-CCTCCAAATCCAAGACTCCAGCAG; R, 5'-GGAGGAGGAGGAGCTTTGGGACAAG; 28 cycles); *ODC* (accession no. X56316) (F, 5'-GTCAATGATGGAGTGTATGATC; R, 5'-TCCATTCCGCTCTCCTGAGCAC; 28 cycles). UV and LiCl treatment were as described¹⁴. Whole-mount procedure is described in Fig. 2.

(Fig. 2b, c) does not extend into the dorsal lip, which is marked by *chd*, although an area of overlap can be seen in histological sections (Fig. 2b). At mid-gastrula (stage 11), *cerberus* expression is suppressed in the dorsal midline in a region that appears to correspond to prechordal plate precursor cells (Fig. 2d). To substantiate this observation we compared the expression domains of *cerberus* (dorso-anterior yolk endomesoderm), *Xbra* (ref. 31; trunk-tail mesoderm), *Xlim-1* (ref. 3; prechordal and notochordal precursors), *gooseoid* (ref. 2; prechordal plate), *Xnot-2* (ref. 7; notochord) and *chd* (ref. 14; prechordal and notochordal cells) in a double-labelled *in situ* hybridization study. The *cerberus* midline gap does not correspond to notochordal tissue because *Xbra* and *Xnot-2* do not overlap with it (Fig. 2d, h). Conversely, this gap coincides with markers expressed in prechordal plate precursors such as *Xlim-1* (Fig. 2e), *gooseoid* (Fig. 2f, g) and *chd* (Fig. 2i). The patterns are highly dynamic; for example, at early gastrula, *gooseoid* and *Xlim-1* extend to the floor of the blastocoel, overlapping extensively with *cerberus* (not shown), whereas at mid-gastrula they separate from each other, so that *cerberus* occupies the anteriormost endomesoderm (Fig. 2c), and *gooseoid* and *Xlim-1* become restricted to prospective prechordal plate (Fig. 2g and data not shown). Within the mid-gastrula stage there are subtle differences in expression domains. We note that *Xlim-1* and *chd* have wider domains than *gooseoid* (Fig. 2e, f, i), resulting in areas of overlap with *cerberus*-expressing cells in the lateral margins. At early neurula (stage 13), *cerberus* mRNA is found in a broad anterior domain of endomesoderm that excludes the prechordal plate before becoming undetectable by stage 14 (not shown).

As summarized in Fig. 2j, these comparative *in situ* hybridization studies show that the endomesoderm of the dorsal side of the gastrula can be subdivided into three main regions: (1) the future trunk-tail mesoderm defined by *Xbra*; (2) the prospective prechordal plate that expresses *chd*, *gooseoid* and *Xlim-1*; and (3) a broad anterior domain of *cerberus*-expressing cells that includes the leading edge of the gastrulating endomesoderm.

To investigate whether *cerberus* expression is controlled similarly to Spemann's organizer activity, we analysed its expression in ultraviolet-treated embryos. This treatment (Fig. 1c, d), which

prevents the induction of Spemann's organizer²⁴, was found to inhibit *cerberus* strongly. Conversely, when the amount of Spemann's organizer is increased by treatment with LiCl²⁴, *cerberus*, like *chd*, is upregulated (Fig. 1c). We therefore tested whether *cerberus* can be activated by signals secreted by the organizer. Embryos were injected into each of the four blastomeres with *chd* (Fig. 1d), *noggin* or *folliculin* (not shown) mRNAs, all of which expanded expression of *cerberus* radially in the endomesoderm. The results suggest that *cerberus* delineates a novel anterior domain of the *Xenopus* gastrula that is controlled in a similar way to Spemann's organizer.

Prospective foregut marked by *cerberus*

The population of *cerberus*-expressing cells at the leading edge of the gastrulating endomesoderm (Fig. 2c) has been poorly defined in *Xenopus*. Apart from experiments in which explants of this region gave rise to liver³², little is known about the fate of these anteriormost gastrulating cells. We labelled the leading edge of these cells with the hydrophobic lineage tracer DiI (Fig. 2k) to determine their fate, and cultured the embryos until stage 30. In 85% of the cases ($n = 47$, two experiments) the lineage tracer marked the large endodermal cells of the foregut, particularly in the liver diverticulum, and the anterior midgut (Fig. 2l). Contrary to our expectations, we did not observe labelled cells in the prechordal plate, head mesoderm or pharyngeal endoderm. In hindsight, the fate of the leading-edge cells could have been predicted from detailed examination of published histological sections of *Xenopus* embryos^{28,33}. Lateral to the midline, the *cerberus*-positive region becomes heart primordium³⁴. We conclude from this cell-lineage study that the dorsal leading edge of the gastrula gives rise to foregut, liver and midgut.

Suppression of trunk-tail mesoderm formation

Microinjected *cerberus* mRNA has potent effects on *Xenopus* development. Radial injection of high doses (100 pg per blastomere) of *cerberus* mRNA into each blastomere of the four-cell embryo results in embryos with very large cement glands (Fig. 3a). These embryos do not form trunk-tail mesoderm, as indicated by the repression of *Xbra* (Fig. 3b), the lack of an external blastopore

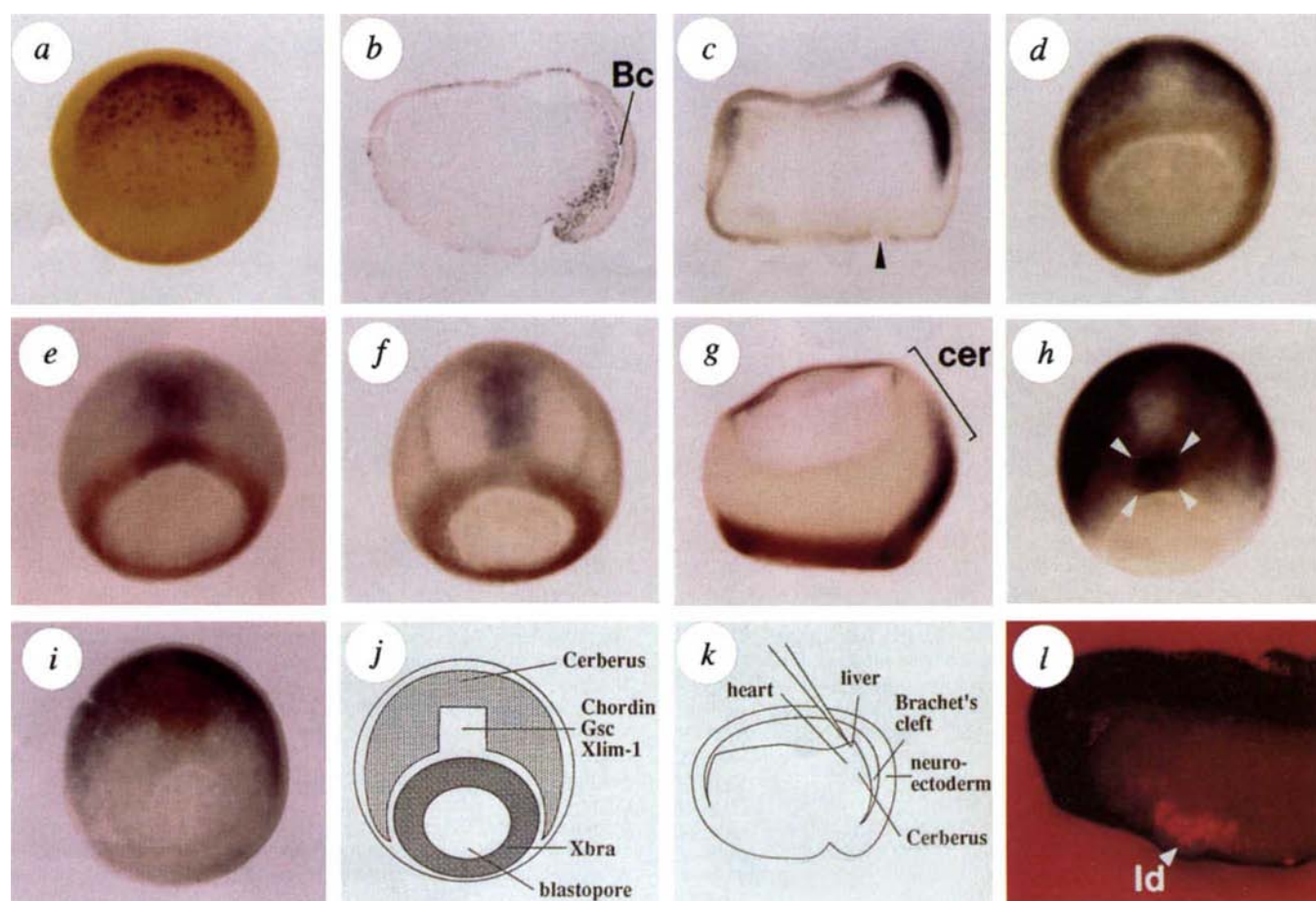


FIG. 2 The *cerberus* (*cer*) expression domain and comparison with other organizer genes. **a**, Top view at stage 10 $\frac{1}{2}$, showing *cer* expression in the dorsal half of the deep endomesodermal cells and the floor of the blastocoel. **b**, Histological section of a stage-10 $\frac{1}{2}$ gastrula stained with *cer* (blue) and *chd* (brown); *chd* is expressed in the dorsal lip, *cer* in the anterior endomesoderm (separated from the neuroectoderm by Brachet's cleft, Bc). Note the overlap between the two areas of expression. **c**, Lateral view of *cer* at stage 10 $\frac{3}{4}$ marking the anteriormost endomesoderm. The *cer* domain does not extend to the blastopore (arrowhead). **d**, Double staining of mid-gastrula (stage 11) embryo for *cer* (blue) and *Xbra* (brown); *cer* expression is anterior to *Xbra*, which forms a ring defining the trunk-tail mesoderm region. Note that *cer* expression is suppressed in the midline region corresponding to the future prechordal plate. **e**, Double staining (stage 11) for *Xlim-1* (blue) and *Xbra* (brown). **f**, Comparison of *gsc* (blue) and *Xbra* (brown), dorsal view. **g**, Lateral view of *goosecoid* (*gsc*) and *Xbra* double labelling. At stage 11, *gsc* (blue) does not reach the leading edge. The region corresponding to *cerberus* expression in the anteriormost endomesoderm is indicated. **h**, Comparison of *cer* and *Xnot-2*, both blue. Arrowheads mark *Xnot-2* notochordal expression. The prechordal plate gap is not stained. **i**, Double staining (stage 11 $\frac{1}{2}$) for *cer* (blue) and *chd* (brown) showing that *chd*, which marks the prechordal plate at this stage, occupies the *cer* gaps and overlaps with *cer* in lateral regions. **j**, Schematic

representation of a mid-gastrula embryo showing the three main subdivisions of the dorsal side of the *Xenopus* endomesoderm suggested by comparative double-labelling studies. The anterior endomesoderm expresses *cer*; the prechordal plate region expresses *chd*, *gsc* and *Xlim-1*; the trunk-tail mesoderm expresses *Xbra*. **k**, Design of lineage tracing experiment. Schematic diagram showing a lateral view of an early gastrula indicating the injection of the Dil tracer through a cut in the animal cap; *cerberus* is expressed in cells fated to become liver and cardiac primordia. **l**, Side view of a tailbud embryo (stage 30), with anterior to the left, showing labelling in the descendants of the injected leading-edge cells. Fluorescent cells can be identified as endodermal owing to their large size, and are located in the foregut, liver diverticulum (ld) and anterior midgut.

METHODS. Whole-mount *in situ* hybridization was performed as described^{14,27}, using a pBS-*cer* plasmid containing a 1.4-kb insert linearized with *EcoRI* and transcribed with T7 RNA polymerase. Double-labelling procedure was as described⁴⁸; *cer*, *Xlim-1* (ref. 3), *gsc* (ref. 2) and *Xnot-2* (ref. 7) were labelled with digoxigenin-UTP, whereas *chd*¹⁴ and *Xbra*³¹ probes were labelled with fluorescein-UTP. Dil labelling⁷ was done by injecting the dye through a cut in the blastocoel roof (which heals rapidly) into the midline of the leading edge of the crawling endomesodermal yolk cells of stage-10 $\frac{3}{4}$ embryos. Lineage-traced embryos were incubated in the dark until stage 30, fixed and photographed⁷.

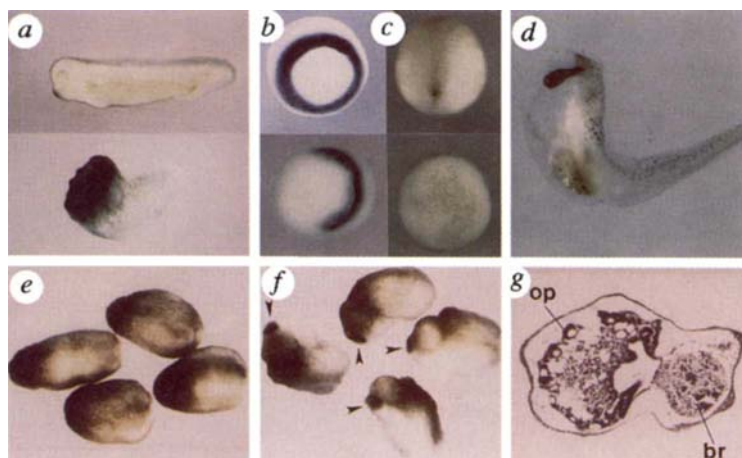
lip, even at stage 13 (Fig. 3c), and by several molecular markers (Fig. 3h).

Radial injection of *cerberus* mRNA also affects prechordal plate mesoderm. This is reflected in the appearance of cyclopic eyes, particularly in embryos injected radially with low doses (20 pg) of *cerberus* mRNA (Fig. 3d). During amphibian development, the prospective eye region is initially specified as a single field that is subsequently split into two eye primordia by signals emanating from the prechordal plate³⁵. Thus *cerberus*, a gene not expressed in the prechordal plate (Fig. 2j), seems to repress prechordal plate function when misexpressed; this view is supported by the ability of *cerberus* to suppress *goosecoid* expression in *in situ* hybridizations at the early neurula stage (data not shown).

Axial development can be restored in embryos ventralized by

ultraviolet treatment by several organizer-specific genes^{8,12,14,17,26}. However, microinjection of *cerberus* mRNA into single blastomeres of eight-cell ultraviolet-treated embryos did not lead to the rescue of trunk-tail axial structures; instead it promoted formation of a large endodermal mass and a smaller head-like structure containing single cement glands and, at later stages, a small cyclopic eye (Fig. 3e, f). To eliminate the possibility that this phenotype could be due to an overdorsalization of the embryo, we performed concentration curves (20, 40 and 80 pg per embryo, $n = 40$), and found no evidence of trunk-tail structures, even at the lowest mRNA concentration (in which only cement glands were observed); this interpretation was supported by molecular analysis (not shown). Histological analysis of the head-like structures revealed the presence of differentiated brain tissue, recog-

FIG. 3 Injection of *cerberus* mRNA into 4-cell embryos suppresses axial mesodermal structures and induces anterior neural structures in UV-ventralized embryos. **a**, Control stage 27 (top), and embryo injected radially into each blastomere at the 4-cell stage with 100 pg pBS-*cer* mRNA (bottom), showing presence of pronounced cement gland and absence of axial trunk–tail structures. **b**, Expression of *Xbra* in control stage 11 (top) and in an embryo injected laterally with *cerberus* mRNA (bottom). Note that *cerberus* suppresses *Xbra* and blastopore lip formation in half the embryo. **c**, Control stage 13



(top) and vegetal view of a radially *cer*-injected sibling lacking the blastopore (bottom). **d**, Embryo injected radially with low (20 pg per blastomere) pBS-*cer* mRNA, leading to formation of a cyclopic eye. **e**, UV-control embryos showing complete ventralization. **f**, UV-injected embryos (single injection of 40 pg pBS-*cer* mRNA at the 8-cell stage, $n = 20$) showing induction of small head-like structures containing cement glands (arrowheads). No rescue of axial structures was observed at lower or higher doses. **g**, Histological section through a UV *cerberus* injected embryo indicating the brain (br) and morphologically recognizable dense olfactory placodes (op); (see Fig. 5c top inset for normal olfactory placode). **h**, Molecular analysis by RT-PCR for expression of gene markers in radially injected embryos and in VMZ-injected explants. Controls (Wt, stage 27), VMZ, LMZ (explants devoid of organizer tissue) and DMZ are controls (see text). Expression of mesodermal (*collagen II*, α -actin, α -globin), cardiac (*Nkx-2.5*), neural (*N-CAM*) and cement gland (*CG-13*) gene markers were analysed. *EF-1 α* is a loading control.

METHODS. Capped synthetic mRNA was produced from two sources: pBS-*cer* (original isolate in pBluescript SK⁺ linearized with *Xho*I, transcribed with T3) or pSP35-*cer* (construct with β -globin 5' and 3' flanking sequences⁴⁰, linearized with *Not*I and transcribed with SP6) using the

Ambion Message Machine kit. To construct pSP35-*cer*, the coding region of *cerberus* was amplified by PCR using the following oligos: (F, 5'-ACGGAATT-CACAATGTTACTAAATGTAATCTCAGG and R, 5'-TGCGTCGACCTTAATGGTG-CAGGGTAGTAGATGT), and cloned into the *Eco*RI and *Sall* sites of pSP35 (ref. 40). Radial injections were performed in the equator region of each blastomere of a 4-cell embryo, at concentrations ranging from 100 to 10 ng μ l⁻¹ RNA synthesized from both pBS-*cer* and pSP35-*cer*. Similar results were obtained with both mRNAs, except that pSP35-*cer* was as active at lower dilutions (about 2 times). Control injections of pBS- Δ *cer* (in which a small deletion was introduced between nucleotides 346 and 566 and that results in a frameshift) had no phenotypic effect in these and other assays. Cyclopic phenotypes were also obtained with a pCS2-*cer* (CMV promoter-driven) DNA construct (not shown). UV embryos were obtained as described⁴⁴ and injected into a single vegetal blastomere at the 8-cell stage. Primer sets for RT-PCR were previously described¹⁵ except for the following new sets: *Nkx-2.5* (F, 5'-GAGCTACAGTTGGGTGTGTGTGGT and R, 5'-GTGAAGCGACTAGGTATGTGTTC; 28 cycles³⁴); *Edd* (F, 5'-TATTCTGACTCCT-GAAGGTG, and R, 5'-GAGAACTGCCATGTGCCTC; 28 cycles⁴⁰). Whole-mount *in situ* hybridization²⁷ was performed with antisense *Xbra* RNA³¹.

nizable by the presence of grey and white matter and, connected to the brain, large masses of tissue with the histological appearance of multiple olfactory placodes (Fig. 3g; see Fig. 5c top inset for a normal *Xenopus* olfactory placode). Thus *cerberus* mRNA differs from previously described organizer factors in that it promotes formation of cement gland and anterior neural structures, but does not rescue axis formation in embryos ventralized by ultraviolet treatment.

To confirm that mesoderm formation is suppressed in radially injected embryos, and that *cerberus* mRNA is unable to dorsalize ventral mesoderm, we analysed several molecular markers by reverse transcription polymerase chain reaction (RT-PCR) analysis (Fig. 3h). RNAs from stage-27 uninjected embryos (Wt), ventral marginal zone (VMZ), lateral marginal zone (LMZ, lacking the organizer region) and dorsal marginal zone (DMZ) explants were used as controls. In radial injections, *cerberus* mRNA abolished the expression of notochordal (*collagen II*), somitic (α -actin) and ventral (α -globin) trunk mesoderm markers. In VMZ explants, *cerberus* mRNA did not cause dorsalization (that is, *collagen II* and α -actin were not induced) and repressed ventral mesoderm (α -globin was inhibited). Instead, in these VMZ explants *cerberus* induced the expression of neural (*N-CAM*), cement gland (*CG-13*) and heart primordium (*Nkx-2.5*) (ref. 34) markers.

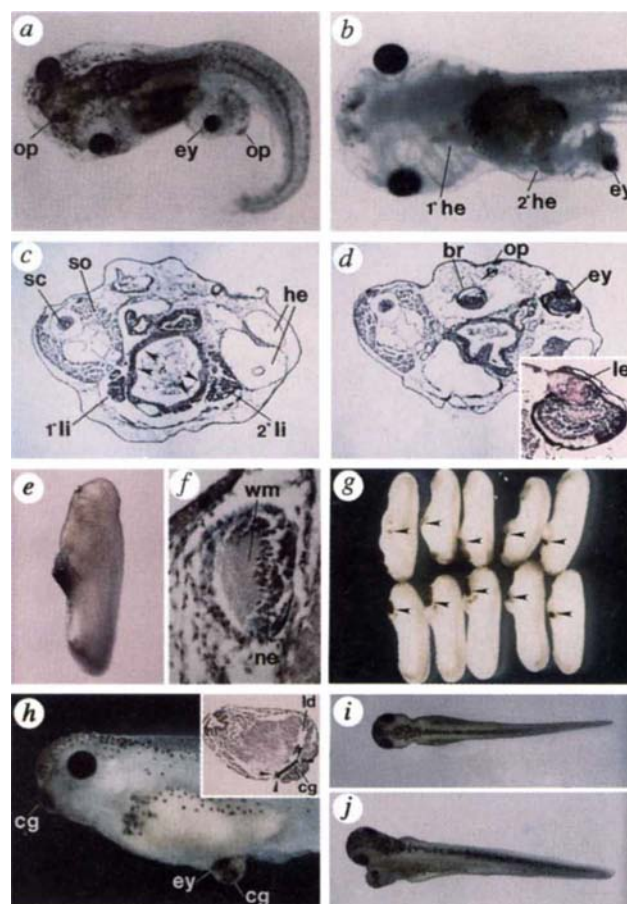
These data indicate that *cerberus* has biological activities that differ from all other known organizer-specific genes. It suppresses trunk–tail mesoderm, promotes cyclopia, presumably by inhibiting formation of the prechordal plate, and induces anterior neuroectodermal structures such as brain, olfactory placodes and cement gland.

Induction of ectopic head structures

To test for more localized effects of *cerberus* mRNA, we injected it systematically into individual blastomeres at the 32-cell state³⁶. In dorsal cells (B1, C1 and D1), *cerberus* mRNA resulted in severe gastrulation defects, and in animal cells (A1, A4) it induced ectopic cement glands. However, striking results were found after injection into the D4 (and to a lesser extent into C4) ventral–vegetal blastomere, which led to the formation of small ectopic head structures (Fig. 4a). These secondary heads were mirror duplications with respect to the anteroposterior axis, and did not contain any trunk–tail axial structures such as somites or notochord (Fig. 4a). In every case in which ectopic eyes developed (scored at 4 days of development), the ectopic head contained only one eye ($n = 45$), suggesting that the secondary heads lack a prechordal plate³⁵. In addition, some of these animals ($n = 21$) had a secondary heart that could be seen beating at a rhythm different to that of the primary heart (Fig. 4b). Histological sections through the duplicated heart region (Fig. 4c) show that the heart consists of atrium and ventricle. Furthermore, a secondary liver (composed of sheets of basophilic hepatocytes flanked by sinusoid capillaries) is attached to the gut, and duplications in epithelial morphology (arrowheads) can be seen in the gut (Fig. 4c). The secondary liver arises through the formation of a second liver diverticulum, which was observed frequently in sections of stage-27 injected embryos (found in all seven embryos sectioned; not shown). Histological sections through more posterior regions (Fig. 4d) show that the ectopic eye has a lens and the typical retinal layered structure (Fig. 4d, inset). The cyclopic eye is connected to an ectopic brain containing white and grey matter, which is connected to multiple olfactory placodes (Fig. 4d).

FIG. 4 Ectopic *cerberus* expression at the 32-cell stage induces head structures and duplicated heart and liver. **a**, Four-day-old embryo, anterior to the left, injected into a single D4 blastomere, showing an induced secondary head with cyclopic eye (ey) and olfactory placode (op). **b**, Ventral view of another injected embryo with ectopic head and eye (ey) and a second functional heart (2° he) containing circulating blood cells. **c**, Transverse section through the level of the ectopic heart. Note the secondary heart (he), with a ventricle and atrium as well as secondary liver (2° li) that is attached to a gut showing traces of duplicated epithelial differentiations (arrowheads); sc, spinal cord; so, somite. **d**, Transverse histological section through the level of the ectopic eye. Note the cyclopic eye (ey) and secondary brain (br) containing white and grey matter, as well as olfactory placodes (op). This particular embryo had up to eight nasal placodes. Inset, magnification of the ectopic eye, showing the typical appearance of retinal epithelium, photoreceptor cells and crystalline lens (le). **e**, Lineage-traced ectopic head structure; most *cer*-injected cells have migrated into the induced structure. **f**, Section through a lineage-labelled (*lacZ*) ectopic brain structure at day 3. Labelled cells tend to occupy the periphery of the neural tissue; wm, white matter; ne, neural. **g**, Ectopic induction of anterior structures and cement glands (arrowheads) in stage-27 embryos that were produced by transplantation into the blastocoel⁷ of small animal cap fragments of *cer*-injected embryos. Control caps did not cause any induction. **h**, Ectopic head induced by transplantation of *cer*-injected animal cap cells at the gastrula stage. Note the presence of cement gland (cg) and a cyclopic eye (ey). Inset, histological section through a lineage-traced embryo at stage 27. Host cells are recruited into the cement gland (cg) and liver diverticulum (ld) and the graft (demarcated by arrowheads) is intensely labelled by the *lacZ* lineage tracer. **i**, Three-day-old embryo that received a graft of leading edge endomesoderm; no induction was found. **j**, Embryo that received a graft of more posterior endomesoderm (from the base of Brachet's cleft) where *cer* and *chd* overlap; a secondary head-like structure was formed.

METHODS. Single blastomere injections were performed in a volume of 2 nl, at a concentration of $25 \text{ ng } \mu\text{l}^{-1}$ of pSP35-*cer* mRNA. Of all embryos injected ($n = 185$, 6 independent experiments) into D4 blastomeres, 75% produced ectopic patches of cement-gland tissue. Ectopic small heads with fully developed cyclopic eyes were induced at frequencies of about 35% (depending on the batch) of the embryos allowed to develop until day 4. Secondary hearts were formed in 46% of embryos with well-formed secondary heads. For the Einsteck transplantation procedure, the four animal blastomeres of 8-cell embryos were injected with 200 pg pBS-*cer* mRNA and cultured until stage 10. Animal caps were explanted and a small fragment (about $\frac{1}{4}$ of the cap) was transplanted into the



blastocoelic cavity of recipient embryos at stage 10 $\frac{1}{2}$. For endomesoderm transplantation, transplanted cells were explanted and cultured for at least 30 min in $0.3 \times$ Barth's to allow aggregation. Each graft filled about half of the blastocoel cavity. Histological analysis was performed as described⁴⁰.

To determine whether *cerberus* can induce ectopic structures on neighbouring cells, we co-injected *cerberus* and *lacZ* mRNAs into D4 blastomeres. The injected cells were found predominantly in the secondary head region (Fig. 4e), with some labelled cells trailing back towards the anus. Because the normal fate of D4 is to form posterior endoderm and mesoderm³⁶, the observed anterior shift suggests that *cerberus* overexpression may cause cells to migrate in the anterior direction, a change in cell behaviour that has been noted previously in *gooseoid* injections³⁷. When sectioned, the labelled cells were found predominantly in the periphery of organ differentiations such as brain (Fig. 4f), cement gland, liver diverticulum and placode. However, some labelled cells can also contribute to all of the organs induced by *cerberus*. Although these experiments do not demonstrate a particular fate for *cerberus*-injected cells, they show that non-injected neighbouring cells are recruited into the ectopic head structures. To test whether cell recruitment can also occur at the gastrula stage, we transplanted animal caps injected with *cerberus* mRNA, together with *lacZ* mRNA as a lineage marker, into the blastocoel of stage-10 embryos (the Einsteck procedure). The transplanted cells induced head-like structures with cement glands (arrows in Fig. 4g) at high frequency (72%, $n = 46$, two experiments). When allowed to develop until day 4, head-like structures with cyclopic eyes were formed (Fig. 4h). Staining for β -galactosidase activity at stage 27 and sectioning showed that labelled grafted cells (arrowheads in Fig. 4h inset) were able to recruit host cells into cement gland and secondary liver diverticulum. Thus *cerberus* protein has non-cell-autonomous effects on the differentiation of anterior tissues.

In an attempt to test the inductive activity of the endomesoderm we transplanted the yolk cells of the leading edge of the stage-11 gastrula by the Einsteck procedure. We found that this region largely lacked inductive activity (only 2 of 42 embryos had head-like structures, two experiments). We then compared the inductive activity of the dorsal leading-edge endomesoderm with that of the more posterior endomesoderm located closer to the base of Brachet's cleft (Fig. 2b). Although the leading edge lacked inductive activity (Fig. 4i, $n = 9$), the endomesoderm just posterior to it induced head-like structures in the absence of trunk-tail differentiations (Fig. 4j; 8 ectopic head-like structures, 2 ectopic cement glands, $n = 18$). These observations suggest that in *Xenopus* the head organizer activity resides in the endomesoderm located at the base of Brachet's cleft, where the expression of *cerberus* overlaps with that of *chd*, *gooseoid* and *Xlim-1* (Fig. 2b). The lack of inductive activity of the leading edge could be affected by the experimental procedure, as the yolk explant of large cells must be allowed to aggregate for at least 30 min in saline before transplantation is possible. The interpretation we favour is that the maintenance of *cerberus* expression requires signals from the classical organizer, as suggested by the ability of *chd*, *noggin* and *folliculin* to upregulate *cerberus*, and by the lack of *cerberus* expression in ultraviolet-treated embryos (Fig. 1d).

The results indicate that *cerberus* has inductive activities on neighbouring cells, leading to the formation of ectopic heads that contain forebrain, cyclopic eyes, nasal placodes, and duplicated internal organs such as heart and liver. The *in vivo* expression of *cerberus* might be dependent on the activity of other organizer factors.

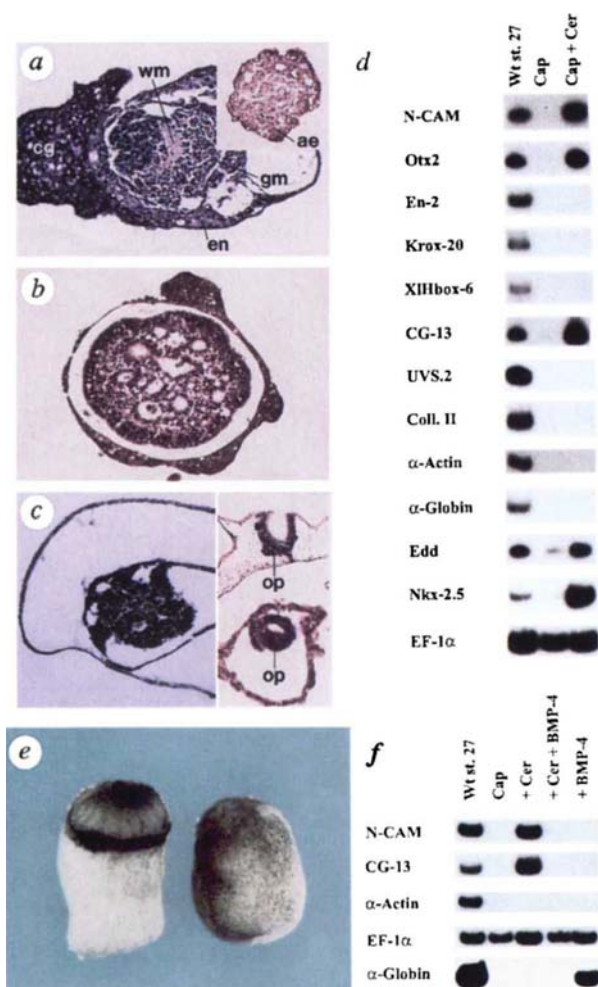


FIG. 5 *cerberus* inductions in animal cap explants. **a**, Histological section of an animal cap cultured for 4 days showing induction of brain with grey matter (dense basophilic neural cells, gm) and patches of white matter (myelinated axons, wm). Cement gland (cg) and endoderm-like cells (en) are indicated in this explant cultured for 4 days. Inset, control cap showing atypical epidermis (ae). **b**, Massive neuroepithelial differentiation of an origin difficult to determine by histological criteria. **c**, Section showing very dense neural cells that can be recognized morphologically as olfactory placodal cells. Top inset, control olfactory placode (op) of a wild-type 4-day-old embryo. Bottom inset, magnification of olfactory placode (op) induced by *cer* in an animal cap. **d**, Molecular analysis by RT-PCR of induction of anterior neural markers and other gene markers in caps injected with *cer* (see text). **e**, Embryo injected radially with 100 pg *cer* mRNA (left) or with *cer* mRNA plus 50 pg of pCSKA-*bmp-4* DNA¹⁵ (right). Siblings were at stage 27. **f**, Molecular analysis by RT-PCR of the repression of neural and cement-gland markers by *bmp-4* in animal caps injected with *cer* mRNA. **METHODS**. Synthetic *cer* RNA pBS-*cer* (100 pg) was injected into each of the animal cells of 8-cell embryos. Animal caps were explanted at stage 9–10 and grown in culture until siblings reached stage 27 for molecular analyses, and for 4 days for histological sections. Conditions for RT-PCR analysis were as described¹⁵. Results shown are representative of several independent experiments.

Activity of *cerberus* in animal caps

As a further test of its biological activity, we injected *cerberus* mRNA into animal blastomeres of eight-cell embryos and prepared animal cap explants, which were then cultured and subjected to histological and molecular analysis for the expression of a battery of markers. Sections of *cerberus*-injected animal caps indicate a variety of neural differentiations. Control cells (Fig. 5a, inset) form atypical epidermis, but *cerberus*-injected caps differ-

entiate into brain-like structures (containing clearly demarcated grey and white matter) and sometimes contain a layer of endodermal cells (Fig. 5a). In other cases, massive neuroepithelial differentiations are formed, the histological identity of which remains unknown (Fig. 5b). Less frequently, explants are found that consist exclusively of dense neural vesicles and foldings that can be identified histologically as olfactory placodes (Fig. 5c). We have not observed such a variety of tissue patterns in neural tissue induced by *chd*¹⁵, but in the case of *noggin* dorsoventral patterning of forebrain markers has been reported^{38,39}.

Molecular analysis showed that *cerberus* mRNA strongly induces a pan-neural marker (*N-CAM*) in animal caps (Fig. 5d). The neural tissue induced is of the anterior type, as it expresses a forebrain–midbrain marker (*Otx2*) but not more posterior markers (*En-2*, midbrain–hindbrain junction; *Krox-20*, hindbrain; *XlHbox-6*, spinal cord). A cement-gland marker (*CG-13*) is up-regulated, whereas a hatching-gland marker (*USV.2*) is not induced; both organs are ectodermal derivatives. The neuralization by *cerberus* occurs in the absence of trunk mesoderm formation (*collagen II*, α -actin or α -globin). Furthermore, *cerberus* can induce the pan-endodermal marker *endodermin*⁴⁰ (*edd*), as well as high levels of an early homeobox marker for prospective heart mesoderm³⁴ (*Nkx-2.5*). It has been proposed that the neural inducing activities of *chd*, *noggin* and *follistatin* might be the result of antagonizing endogenous bone morphogenetic protein-4 (*Bmp-4*) signalling in animal cap explants⁴¹. To test whether *Bmp-4* can override *cerberus* signalling, we co-injected *cerberus* mRNA and pCSKA-*bmp-4* DNA. *Bmp-4* was able to counteract the morphological effects of *cerberus* radial injection (Fig. 5e) and the induction of *N-CAM* and a cement-gland marker in animal caps (Fig. 5f). Thus the antineurogenic effect of *Bmp-4* is dominant over *cerberus* protein. This effect is not due to a shift of animal cap cells to ventral mesodermal fates because α -globin, which is activated by *Bmp-4* alone, is repressed by co-injection of *cerberus* mRNA (Fig. 5f). Because *Bmp-4* can counteract the effects of *cerberus* on ectoderm, but *cerberus* is dominant over the ventral mesoderm inductive activity of *Bmp-4*, the results suggest that *cerberus* and *Bmp-4* signal through different pathways. We conclude that *cerberus* mRNA leads to the formation of a variety of differentiated anterior neural tissues, as well as to formation of endoderm and activation of a prospective heart mesoderm marker in animal caps.

Discussion

The cDNA *cerberus* encodes a secreted protein that is transiently expressed in a broad anterior domain of the gastrula. This domain includes the leading edge of the involuting endoderm, which, as shown here, gives rise to foregut, liver and anterior midgut. More laterally, the *cerberus* domain includes the cardiac primordia³⁴. Expression of *cerberus* is excluded from the prospective prechordal plate region and from the ring of *Xbra*-expressing cells that give rise to the trunk–tail mesoderm. The biological activity of microinjected *cerberus* mRNA is congruent with this subdivision of the dorsal side of the embryo into three main regions (Fig. 2j). Ectopic *cerberus* mRNA inhibits prechordal plate function, leading to cyclopia, and can completely suppress formation of trunk–tail mesoderm, inhibiting notochordal, somitic and ventral mesoderm markers. Ultraviolet-ventralized embryos cannot be dorsalized by *cerberus*, which promotes anteriorization of the embryo instead. When misexpressed in a ventral–vegetal blastomere at the 32-cell stage, *cerberus* induces the formation of ectopic head structures, as well as duplications of internal organs derived from anterior endomesoderm, in particular heart and liver.

The domain of *cerberus* expression is wider than the classical Spemann organizer region defined by transplantation experiments, which occupies only 60° of the dorsal blastopore⁴². However, *cerberus* expression is dependent on the presence of a functional organizer, as ventralization by ultraviolet treatment abolishes its expression. Conversely, dorsalization by LiCl leads to radial *cerberus* expression. It is possible that *cerberus* expression

TABLE 1 *Xenopus* dorsal-specific cDNAs isolated by differential screening

Previously known genes	Gene product	No. of isolates
<i>chordin</i>	novel secreted protein	70
<i>goosecoid</i>	homeobox/transcription factor	3
<i>pintailavis/XFKH-1</i>	forkhead/transcription factor	2
<i>Xnot-2</i>	homeobox/transcription factor	1
<i>Xlim-1</i>	homeobox/transcription factor	1
New genes		
<i>cerberus</i>	novel secreted protein	11
<i>PAPC</i>	cadherin-like/transmembrane	2
<i>frezzed</i>	secreted protein	1
<i>Sox-2</i>	sry/transcription factor	1
<i>Fkh-like</i>	forkhead/transcription factor	1

Subtractive differential screening was performed essentially as described¹⁴. In brief, poly(A)⁺ RNA was isolated from 300 dorsal lip and ventral marginal zone (VMZ) explants at stage 10^{1/2}. After first-strand cDNA synthesis, ~70–80% of common sequences were removed by subtraction with biotinylated VMZ poly(A)⁺ RNA prepared from 1,500 ventral gastrula halves. For differential screening, duplicate filters (2,000 plaques per 15 cm plate; 80,000 clones screened) of an unamplified oriented dorsal lip library⁴⁶ were hybridized with radiolabelled dorsal lip or VMZ cDNA. Putative organizer-specific clones were isolated, grouped by sequence analysis from the 5' end and whole-mount *in situ* hybridization, and classified into known and new dorsal-specific genes. Rescreening of the library (100,000 independent phages) with a *cerberus* probe resulted in the isolation of 45 additional clones; 31 were similar in size to the longest of the 11 original clones, indicating that they were presumably full-length cDNAs. The two longest cDNAs were completely sequenced.

may be maintained by diffusible signals such as *chordin*¹⁴, *noggin*¹² or *folliculin*^{13,15}, all of which can activate its transcription in microinjected embryos. This could provide an explanation for the absence of head-inducing activity of the leading edge of the endomesoderm in the Einsteck experiments reported here, and the failure of the anterior endoderm (at a later stage) to induce cement gland and neural tissue in conjugates⁴³. In both cases the inducing activity was found in more posterior regions of the endomesoderm, where the expression of *cerberus* and *chordin* overlap.

The *cerberus* mRNA is a potent neuralizing agent in injected animal caps, although we have not yet obtained purified *cerberus* protein to test whether this is a direct or indirect neural induction. Ectoderm is neuralized by *cerberus* mRNA in the complete absence of trunk–tail mesoderm (marked by *collagen II*, α -*actin* and α -*globin*), as is also the case for the neural inducers *noggin*³⁸, *folliculin*¹³ and *chd*¹⁵. The panendodermal marker *endoderm* and the prospective heart mesoderm marker *Nkx-2.5* are also induced by *cerberus*. At present, therefore, we cannot determine whether *cerberus* protein is a direct neural inducer or whether it requires an intermediate signal from the endoderm or heart mesoderm. It has recently been reported that *chd* and *noggin*, as well as neural tissue, also induce endoderm in animal cap

explants⁴⁰. The induction of *Nkx-2.5* in animal caps has not to our knowledge been reported previously. Neuralization by *cerberus* is antagonized by *Bmp-4*, as is the case for *chd*, *noggin* and *folliculin*¹⁵. In the ectoderm, *cerberus* could act on the *Bmp-4* signalling pathway or on a parallel one, with the antineurogenic activity of *Bmp-4* being dominant. In the mesoderm, *cerberus* appears to act through a different signalling pathway from that of *chd* and *noggin*, because it suppresses trunk mesoderm, whereas *chd* and *noggin* are potent mesoderm dorsalizing agents^{12,14}.

Expression of *Nkx-2.5*, a marker for the cardiogenic region homologous to *Drosophila tinman*³⁴, is induced by *cerberus* mRNA, but we have been unable to detect beating hearts in explants; in intact embryos, however, functional duplicated hearts can be formed by *cerberus* injection. This suggests that in explants an additional factor may be required for overt cardiac differentiation. In *Xenopus*, there is evidence that heart induction requires at least two separate signals, one from the organizer and another from the endoderm^{44,45}. The isolation of the *cerberus* signalling molecule now provides the opportunity to test its function in combination with signalling proteins such as *chordin*, *noggin*, *folliculin*, *Bmp-4* and others in the genesis of organs such as heart, liver, pancreas and brain *in vitro*.

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- Spemann, H. & Mangold, H. *Wilhelm Roux Arch. EntwMech. Org.* **100**, 599–638 (1924).
- Cho, K. W. Y., Blumberg, B., Steinbeisser, H. & De Robertis, E. M. *Cell* **67**, 1111–1120 (1991).
- Taira, M., Jamrich, M., Good, P. J. & Dawid, I. B. *Genes Dev.* **6**, 356–366 (1992).
- Dirksen, M. L. & Jamrich, M. *Genes Dev.* **6**, 599–608 (1992).
- Ruiz i Altaba, A. & Jessell, T. *Development* **116**, 81–93 (1992).
- von Dassow, G., Schmidt, J. E. & Krimelman, D. *Genes Dev.* **7**, 355–366 (1993).
- Gont, L. K., Steinbeisser, H., Blumberg, B. & De Robertis, E. M. *Development* **119**, 991–1004 (1993).
- Lemaire, P., Garrett, N. & Gurdon, J. B. *Cell* **81**, 85–94 (1995).
- Pannese, M. et al. *Development* **121**, 707–720 (1995).
- Blitz, I. L. & Cho, K. W. Y. *Development* **121**, 993–1004 (1995).
- Zaraisky, A. G. et al. *Development* **121**, 3839–3847 (1995).
- Smith, W. C. & Harland, R. M. *Cell* **70**, 829–840 (1992).
- Hemmati-Brivanlou, A., Kelly, O. G. & Melton, D. A. *Cell* **77**, 283–295 (1994).
- Sasai, Y. et al. *Cell* **79**, 779–790 (1994).
- Sasai, Y., Lu, B., Steinbeisser, H. & De Robertis, E. M. *Nature* **376**, 333–336 (1995).
- Smith, W. C., McKendry, R., Ribisi, S. & Harland, R. M. *Cell* **82**, 37–46 (1995).
- Jones, C. M., Kuehn, M. R., Hogan, B. L. M., Smith, J. C. & Wright, C. V. E. *Development* **121**, 3651–3662 (1995).
- Moos, M., Wang, S. & Krinks, M. *Development* **121**, 4239–4301 (1995).
- Vodicka, M. A. & Gerhart, J. C. *Development* **121**, 3505–3518 (1995).
- De Robertis, E. M. *Nature* **374**, 407–408 (1995).
- Spemann, H. *Wilhelm Roux Arch. EntwMech. Org.* **123**, 389–517 (1931).
- Shawlot, W. & Behringer, R. R. *Nature* **374**, 425–430 (1995).
- Acampora, D. et al. *Development* **121**, 3279–3290 (1995).
- Kao, K. R. & Elinson, R. P. *Dev Biol.* **127**, 64–77 (1988).
- Durston, A. J. et al. *Nature* **340**, 140–144 (1989).
- Steinbeisser, H., De Robertis, E. M., Ku, M., Kessler, D. S. & Melton, D. A. *Development* **118**, 499–507 (1993).
- Harland, R. M. *Meth. Cell Biol.* **36**, 685–695 (1991).

- Nieuwkoop, P. D. & Florschütz, P. A. *Archs Biol., Paris* **61**, 113–150 (1950).
- Pasteels, J. *Archs Biol., Paris* **60**, 235–250 (1949).
- Keller, R. *Meth. Cell Biol.* **36**, 61–113 (1991).
- Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. & Herrmann, B. G. *Cell* **67**, 79–87 (1991).
- Holtfrete, J. *Wilhelm Roux Arch. EntwMech. Org.* **138**, 522–738 (1938).
- Hausen, P. & Riebesell, M. *The Early development of Xenopus laevis* (Springer, Berlin, 1991).
- Tonissen, K. F., Drysdale, T. A., Lints, T. J., Harvey, R. P. & Krieg, P. A. *Dev Biol.* **162**, 325–328 (1994).
- Adelman, H. B. *Q. Rev. Biol.* **11**, 284–304 (1936).
- Dale, L. & Slack, J. M. W. *Development* **99**, 527–551 (1987).
- Niehrs, C., Keller, R., Cho, K. W. Y. & De Robertis, E. M. *Cell* **72**, 491–503 (1993).
- Lamb, T. M. et al. *Science* **262**, 713–718 (1993).
- Knecht, A. K., Good, P. J., Dawid, I. B. & Harland, R. M. *Development* **121**, 1927–1936 (1995).
- Sasai, Y., Lu, B., Piccolo, S. & De Robertis, E. M. *EMBO J.* (in the press).
- De Robertis, E. M. & Sasai, Y. *Nature* **380**, 37–51 (1996).
- Gerhart, J. et al. *Development* **107** (suppl.), 37–51 (1989).
- Sive, H. L., Hattori, K. & Weintraub, H. *Cell* **58**, 170–180 (1989).
- Sater, A. K. & Jacobson, A. G. *Development* **108**, 461–470 (1990).
- Nascone, N. & Mercola, M. *Development* **121**, 515–523 (1995).
- Blumberg, B., Wright, C. V. E., De Robertis, E. M. & Cho, K. W. Y. *Science* **253**, 194–196 (1991).
- Ozaki, T. & Sakiyama, S. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2593–2597 (1993).
- Jowett, T. & Lettice, L. *Trends Genet.* **10**, 73–74 (1994).

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CORRESPONDENCE and requests for materials should be addressed to E.M.D.R. The *cerberus* cDNA sequence has been deposited in GenBank database, accession no. U64831.

Sustained star formation in the central stellar cluster of the Milky Way

E. Serabyn* & Mark Morris†

* California Institute of Technology, 320-47, Pasadena, California 91125, USA

† Department of Astronomy, University of California—Los Angeles, Los Angeles, California 90095, USA

THE central stellar bulge of our Galaxy is commonly thought to consist predominantly of a coeval population of very old ($\sim 10^{10}$ yr) stars^{1–3}. But evidence for young⁴ and intermediate-age stars^{5,6} in the bulge is accumulating. Here we argue that star formation has been occurring in the molecular gas near the centre of the bulge throughout the lifetime of the Galaxy, and that the resulting stellar population is evident as the prominent infrared^{7,8} r^{-2} central star cluster (a cluster whose space density decreases as the square of the distance from the centre). Although our hypothesis is at odds with the standard view that this central cluster is the innermost part of the more extended and ancient bulge^{9–11}, sustained star formation is consistent with both the available observational data and simple models of mass inflow and collisionally induced star formation in this region. Even if the central star-formation rate is relatively modest, it appears that the stellar population of the Galactic bulge has been augmented substantially since its birth.

A prodigious reservoir of dense molecular gas ($\sim 5 \times 10^7 M_{\odot}$) is concentrated in a flattened layer occupying the central few hundred parsecs of our Galaxy^{12–14} (Fig. 1). This central molecular zone (CMZ) is an active region of star formation⁴, with massive star-forming molecular clouds, copious ionized gas and a number of very young stellar clusters present. The current star formation rate in the CMZ¹², $\sim (0.3–0.6) M_{\odot} \text{ yr}^{-1}$, about 10% of the overall galactic rate, is well below rates found in ‘star burst’ galactic nuclei ($\sim (1–100) M_{\odot} \text{ yr}^{-1}$), suggesting that our Galactic nucleus is a ‘slow-burn nucleus’. Without replenishment, continual star formation at the current rate would deplete the CMZ of material in $\sim 10^8$ yr. However, as radial gas inflow resulting from the actions of our Galaxy’s stellar bar (and dissipative processes such as dynamical friction) continually feeds material into the centre^{15–17}, at a rate^{4,16} of $\sim 0.4 M_{\odot} \text{ yr}^{-1}$, continual replenishment of the CMZ is feasible. Thus long-term, low-level star formation at the centre of the Milky Way may occur.

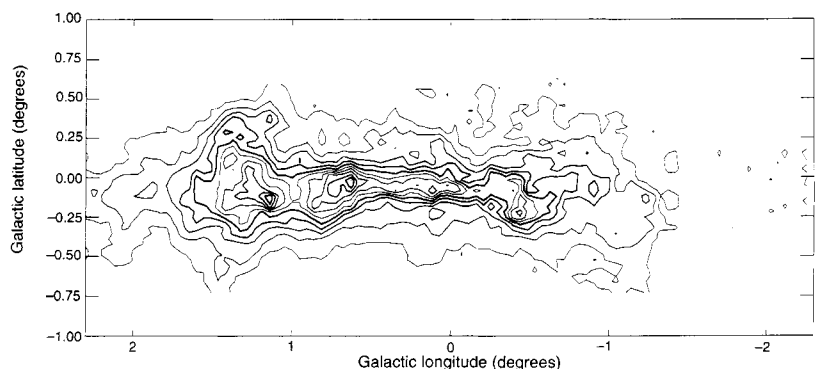
Even low-level star formation in the CMZ would have substantial consequences over the Galaxy’s lifetime: at the current rate of star formation, a total stellar mass of $\sim (3–6) \times 10^9 M_{\odot}$ would have emerged within the CMZ in the past 10^{10} yr. Even allowing that the turbulent Galactic Centre environment may prohibit the formation of lower-mass stars^{12,18}, and that some

CMZ material may be lost to a galactic wind⁴, some $10^9 M_{\odot}$ of stars will still have formed in the CMZ. Such an agglomeration of intermediate-age stars (those born between the formation of our galaxy and the present) should be quite evident as an enhancement in our Galaxy’s central light distribution. Visual observations of the centre of our Galaxy are precluded by the high intervening interstellar extinction, but near-infrared observations^{7,8} have revealed a bright, centrally concentrated stellar cluster occupying roughly our Galaxy’s central degree ($1^{\circ} \equiv 140$ pc for a distance to the centre of 8 kpc). The stellar number density in this cluster falls off with Galactocentric radius, r , roughly as an $r^{-2.0 \pm 0.3}$ power law (azimuthal symmetrization of the flattened distribution^{7,19} yields the oft-quoted exponent of -1.8 , while allowing for the observed ellipticity⁸ yields exponents near -2.3). As r^{-2} is the dependence expected for a relaxed isothermal cluster, the default assumption has been that this nuclear cluster is merely the innermost part of our Galaxy’s elderly central bulge^{9–11}. However, as the more extended, kiloparsec-scale Galactic bulge has been observed in its entirety only with low-resolution satellite observations, while much higher-angular-resolution, ground-based observations seldom cover more than a degree in radius, the ‘merging’ of the ‘central r^{-2} cluster’ and the bulge occurs at a scale quite difficult to observe. Thus, the reality of the single-component bulge concept has not been substantiated, and actually is often introduced merely as a simplification²⁰.

There is, however, ample evidence that the central r^{-2} cluster is a component separate from the bulge. The highest-resolution, large-scale near-infrared bulge profiles available (from measurements covering the bulge with a single instrument^{21,22}; Fig. 2a) show good evidence for a distinct, localized brightness enhancement in the bulge’s central degree. Furthermore, the finer-resolution longitudinal profile²³ (Fig. 2b), while not covering the bulge’s full extent, clearly shows a central emission peak rising above a much weaker and more extended continuum (the bulge). The longitudinal extent of this central peak, roughly 0.7° in radius, agrees reasonably well with the size of the central r^{-2} cluster seen with wide-field, high-resolution, 2.4- μm , ground-based observations⁸, implying that the central peak in all of these near-infrared data sets can be attributed to emission from our galaxy’s central r^{-2} cluster. As this central cluster of ~ 100 pc radial extent is evidently morphologically distinct from the larger-scale bulge, we refer to it hereafter as the ‘nuclear cluster’, in keeping with extragalactic nomenclature. Due to difficulties introduced by high extinction, its makeup remains largely unknown outside of the well studied central parsec, but the presence of at least some young and intermediate age stars^{4,6} within the central hundred parsecs renders untenable the notion that this central cluster consists entirely of an old, bulge-like population.

We therefore propose a new model. First, we note that the longitudinal extent of the nuclear cluster (Fig. 2b) is quite similar to the (average) longitudinal extent of the CMZ (Fig. 1)—both lie in the range 100–210 pc (both sizes have substantial uncertainties). Furthermore, both the CMZ (Fig. 1) and the nuclear cluster⁸ show a significant flattening to the Galactic plane. Thus,

FIG. 1 The distribution of molecular material in our Galactic nucleus, as seen in the CO (1–0) line^{4,13}. This material, comprising our Galaxy’s central molecular zone (CMZ), is observed to be quite asymmetric about the centre. Allowing for Galactic rotation, most of the dense molecular material lies within about 1.5° (210 pc) of the centre.



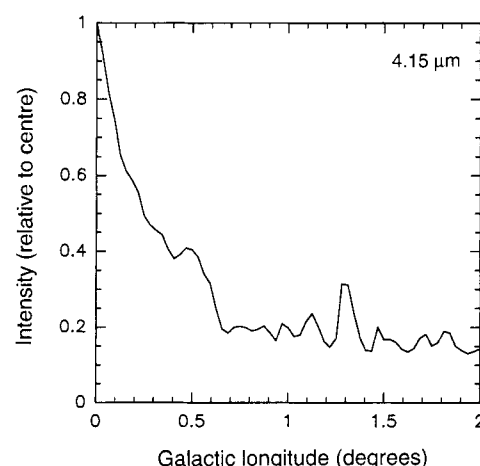
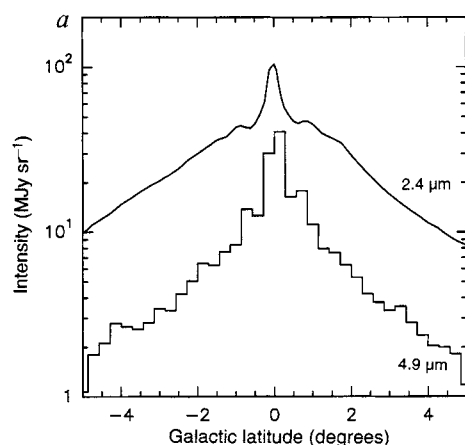


FIG. 2 *a*, Latitudinal intensity profiles across the centre of the Galactic bulge at two wavelengths. The $2.4\text{ }\mu\text{m}$ profile²¹ has the highest spatial resolution available at this wavelength, $0.4''$, and the $4.9\text{ }\mu\text{m}$ COBE data²² have a lower angular resolution, $0.7''$. However, the latter wavelength is less affected by interstellar extinction (which rises toward the Galactic plane, and so might easily suppress, but not enhance, a central peak). The $2.4\text{ }\mu\text{m}$ data is corrected for Galactic-disk emission and extinction, but not for extinction

within the Galactic bulge, while the COBE data are not corrected for extinction. *b*, Higher-resolution ($0.07''$) longitudinal intensity profile (averaging positive and negative longitudes) across the Galactic Centre²³ at a wavelength of $4.15\text{ }\mu\text{m}$. The intensities are normalized by the central value. The r^{-2} nuclear cluster is evident in the central $0.7''$, while the extended low-level emission beyond that radius is attributable to the more extended bulge.

an association of our Galaxy's nuclear cluster with the CMZ is immediately suggested. The mass of the nuclear r^{-2} cluster, probed at radio wavelengths by the kinematics of OH/IR stars⁶ (evolved stars with mass outflow shells emitting strongly in radio OH lines and in the infrared continuum), provides additional support for such an association: the cluster mass, within its $\sim 100\text{-pc}$ extent, is $\sim 10^9 M_\odot$. As this mass falls near the lower end of the range predicted for the stellar mass emerging from the CMZ over the Galaxy's lifetime, and given the dimensional similarities between the CMZ and the observed nuclear cluster, we are led to propose a causal connection. We propose that the observed Galactic ' r^{-2} nuclear cluster' is not part of the old bulge at all, but has instead emerged slowly and steadily from the CMZ via sustained star formation over the lifetime of the Galaxy. The radial extent of the nuclear cluster, being determined in this model by the time-averaged extent of the CMZ, must then ultimately be set by the radius of the Galaxy's inner Lindblad resonance¹⁵⁻¹⁷.

The spectra of stars in the nuclear cluster should then reflect the full range of intermediate and young ages, and the brightest and best-studied individual stars in the central few parsecs are indeed of young or intermediate ages²⁴⁻²⁶. Although a larger observational database is desirable, there is one intermediate-age stellar population which has been directly observed throughout the central few hundred parsecs: OH/IR stars⁶. The similar spatial distributions of the younger (age $\lesssim 10^9\text{ yr}$) subset of these stars and the overall nuclear cluster (both roughly r^{-2} and flattened) supports a common origin, which in the case of OH/IR stars of age $\lesssim 10^9\text{ yr}$ must be star formation over that timescale.

Continuous star formation in dense molecular clouds obviates the relaxed isothermal cluster hypothesis for the nuclear cluster. However, its roughly r^{-2} number density profile may be explicable in terms of the radial run of star formation efficiency. Because of the CMZ's unknown morphology face-on to the Galactic disk, we consider two simple possibilities: a clumpy molecular disk sprinkled uniformly with individual molecular clouds, and a spiral-arm-like geometry akin to the gas distribution²⁷ in the nucleus of IC342. In the first case, molecular clouds spiral inwards owing to dynamical friction and other viscosity sources, and the cloud number density increases as r^{-1} . If star formation is collisionally induced (probably a necessity due to the large shearing forces present in the central region), the rate of star formation would be proportional to the square of the cloud number density, or r^{-2} . In the second case it is reasonable to assume that stars form

along the rotating spiral pattern. The star-formation rate along such a structure is uncertain, but a reasonable guess is that the amount of material swept up at the arms' leading edges varies as r^{-1} , again due to conservation of mass in the inflow. With a radius-independent orbital speed, the frequency of arm passage (that is, of compressional events), is proportional to r^{-1} also. The star-formation rate, proportional to the product of these two factors, is then again $\propto r^{-2}$. A stellar distribution with an r^{-2} profile in the Galactic plane is thus derived in either case (the exact power-law exponent may well not be precisely 2, if, for example, the viscosity or inward velocities have radial dependences). These simple models leave out bar-induced radial streaming motions and orbital resonances, but as the former would tend to bring material into the centre, again leading to a steep power law, while the latter would probably lead to a star formation 'ring' which is not seen, there is little need to introduce these complications.

The initial spatial distribution of stars born in a flattened, rotating molecular layer should be disk-like. The aspect ratio of the resultant nuclear cluster would then be unrelated to that of the bulge. This is supported by extant data, as the r^{-2} cluster^{8,23} is indeed flatter (inner aspect ratio is 2.2, larger in the outskirts) than the bulge²⁸ (aspect ratio 1.7). On the other hand, as the CMZ thickness increases to larger radii¹³, while the star formation rate must increase to smaller radii, a cluster aspect ratio smaller than that of the CMZ (~ 4 ; Fig. 1) is to be expected. Furthermore, the scattering of stars out of their initial orbits by gravitational perturbations such as massive molecular clouds and a central stellar bar²⁹⁻³¹ would inflate the stellar layer with time (thereby also injecting some intermediate-age stars into the bulge.) Observationally, the latitudinal height of the younger OH/IR star layer, 20 pc , is indeed less than that of the older OH/IR stars⁶, and of the infrared nuclear cluster⁸, by about a factor of two.

The kinematics of the emergent intermediate-age stellar population would likewise reflect the initial molecular cloud kinematics, that is, rotation about the centre. In fact, a sizeable rotation component is seen for both red giants and OH/IR stars in the range of several to 100 pc (refs 6, 32). Large line-of-sight, stellar velocity dispersions ($\sim 65\text{ km s}^{-1}$) are also evident outside the central few parsecs—they may reflect (large) initial molecular cloud velocity dispersions¹³ coupled with the action of the aforementioned scattering processes, but line-of-sight rotational broadening also contributes to observed stellar dispersions.

Due to the patchy and intermittent nature of star formation, only the long-term average can yield a smooth r^{-2} number density

profile. At any instant, some young stars should be present at various locations throughout the CMZ, and indeed prolific sites of star formation (for example the 'Arches Cluster' and the Sgr B2 cloud⁴) are found at many projected Galactocentric radii. On the other hand, the steep radial dependence of the star-formation rate implies a high probability of finding young stars in the most central region at any time, suggesting that the central parsec must be the site of either continual star formation, or of frequent, repetitive star-formation bursts. Current evidence tends to favour the latter^{24,25}. In our model, the central parsec is then merely the CMZ's statistically most prolific star-formation site.

A number of questions remain. First, does mass inflow to the nucleus continue over the requisite timescale? The inflow should be stable as long as the driving stellar bar is present, perhaps a few Gyr, but competing models for bar formation, reformation and age render it impossible to answer this question definitively at present. However, a bar even one-third the age of our Galaxy, or multiple accretion events of the Milky Way's nearby neighbours

leading to periodic regeneration of the bar, can provide a time-scale sufficient to generate $10^9 M_{\odot}$ of stars. In any case, dynamical friction, magnetic viscosity and other processes⁴ also engender mass inflow. Of course with an accumulated mass of this size, the nuclear cluster is now at least ~ 3 – 10% as massive as the central stellar bulge/bar⁴, implying that evolution and dynamical interactions between the two are by now significant. Second, are intermediate-age nuclear clusters discernable in the centres of other galactic bulges? While nuclear clusters are indeed present in some galaxies³³, their origins are not yet well constrained. Conversely, while rapid star formation has indeed been inferred for the central regions of many normal (predominantly barred) spiral galaxies³⁴, the large amounts of molecular material in these systems makes the stellar population difficult to observe. Last, is star formation in our own Galaxy's CMZ a slow, continuous process, or is it more episodic and burst-like? Although we do not yet know the answer to this question, we note that even a slow, continuous process could still produce a huge mass of stars. $\square$

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1. Terndrup, D. M. *Astron. J.* **96**, 884–908 (1988).
2. Lee, Y.-W. *Publ. Astron. Soc. Pacif.* **104**, 798–804 (1992).
3. Rich, R. M. in *Galaxy Evolution: The Milky Way Perspective* (ed. Majewski, S. R.) 65–82 (ASP Conf. Ser. Vol. 49, Astr. Soc. Pacif., San Francisco, 1993).
4. Morris, M. & Serabyn, E. *Annu. Rev. Astron. Astrophys.* **34**, 645–701 (1996).
5. Harmon, R. & Gilmore, G. *Mon. Not. R. Astron. Soc.* **235**, 1025–1047 (1988).
6. Lindqvist, M., Habing, H. J. & Winnberg, A. *Astron. Astrophys.* **259**, 118–127 (1992).
7. Becklin, E. E. & Neugebauer, G. *Astrophys. J.* **151**, 145–161 (1968).
8. Catchpole, R. M., Whitelock, P. A. & Glass, I. S. *Mon. Not. R. Astron. Soc.* **247**, 479–490 (1990).
9. Bahcall, J. N., Schmidt, M. & Soniera, R. M. *Astrophys. J.* **258**, L23–L27 (1982).
10. Sellwood, J. A. & Sanders, R. H. *Mon. Not. R. Astron. Soc.* **233**, 611–620 (1988).
11. Evans, N. W. & de Zeeuw, P. T. *Mon. Not. R. Astron. Soc.* **271**, 202–222 (1994).
12. Güsten, R. in *The Center of the Galaxy* (ed. Morris, M.) 89–106 (IAU Symp. No. 136, Kluwer, Dordrecht, 1989).
13. Bally, J., Stark, A. A., Wilson, R. W. & Henkel, C. *Astrophys. J.* **324**, 223–247 (1988).
14. Sodroski, T. J. et al. *Astrophys. J.* **452**, 262–268 (1995).
15. Stark, A. A., Gerhard, O. E., Binney, J. & Bally, J. *Mon. Not. R. Astron. Soc.* **248**, 14p–17p (1991).
16. Gerhard, O. E. *Rev. Mod. Astron.* **5**, 174–187 (1992).
17. Binney, J. in *The Nuclei of Normal Galaxies: Lessons from the Galactic Center* (eds Genzel, R. & Harris, A. I.) 75–86 (Kluwer, Dordrecht, 1994).

18. Morris, M. *Astrophys. J.* **408**, 496–506 (1993).
19. Haller, J. W. et al. *Astrophys. J.* **456**, 194–205 (1996).
20. Kent, S. M. *Astrophys. J.* **387**, 181–188 (1992).
21. Hiromoto, N. et al. *Astron. Astrophys.* **139**, 309–312 (1984).
22. Dwek, E. et al. *Astrophys. J.* **445**, 716–730 (1995).
23. Little, S. J. & Price, S. D. *Astron. J.* **90**, 1812–1819 (1985).
24. Rieke, M. J. in *Back to the Galaxy* (ed. Holt, S. S. & Verter, F.) 37–42 (Am. Inst. Phys., New York, 1993).
25. Krabbe, A. et al. *Astrophys. J.* **447**, L95–L99 (1995).
26. Blum, R. D., DePoy, D. L. & Sellgren, K. *Astrophys. J.* **441**, 603–616 (1995).
27. Turner, J. L., Hurt, R. L. & Hudson, D. Y. *Astrophys. J.* **413**, L19–L22 (1993).
28. Weiland, J. L. et al. *Astrophys. J.* **425**, L81–L84 (1994).
29. Villumsen, J. V. *Astrophys. J.* **290**, 75–85 (1985).
30. Combes, F., Debbasch, F., Freidli, D. & Pfenniger, D. *Astron. Astrophys.* **233**, 82–95 (1990).
31. Jenkins, A. & Binney, J. *Mon. Not. R. Astron. Soc.* **245**, 305–317 (1990).
32. McGinn, M. T., Sellgren, K., Becklin, E. E. & Hall, D. N. B. *Astrophys. J.* **338**, 824–840 (1989).
33. Kormendy, J. in *Galactic Bulges* (eds Dejonghe, H. & Habing, H.) 209–228 (IAU, Symp. No. 153, Kluwer, Dordrecht, 1993).
34. Dressel, L. L. *Astrophys. J.* **329**, L69–L73 (1988).

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CORRESPONDENCE should be addressed to E.S. (e-mail: serabyn@tacos.caltech.edu).

De novo design of structure-directing agents for the synthesis of microporous solids

Dewi W. Lewis*, David J. Willock†, C. Richard A. Catlow*, John Meurig Thomas* & Graham J. Hutchings†

* Davy Faraday Research Laboratory, The Royal Institution of Great Britain, 21 Albemarle Street, London W1X 4BS, UK

† Leverhulme Centre for Innovative Catalysis, University of Liverpool, Liverpool L69 3BX, UK

THE synthesis of microporous materials such as aluminosilicates and aluminophosphates, which are widely exploited as solid acid catalysts, ion exchangers and in gas separation, is facilitated by the use of structure-directing agents (often referred to as templates)¹. These are generally organic bases, and are included in an inorganic gel medium so that the microporous framework condenses around them; the final structure of the framework reflects, to differing degrees, the shape of the template. Although there is growing confidence² in being able to target a particular microporous structure by adroit choice of structure-directing agent^{3–7}, no *a priori* method has been described for generating potential templates for either existing or hypothetical structures. Here we present a method for *de novo* design of template molecules, which are computationally 'grown' in the desired

inorganic framework. Our method is successful in generating known templates for existing microporous materials, and a new candidate suggested by this method succeeds as a template for the target material. Our method should be generally applicable to the field of template-directed hydrothermal synthesis of crystals and to other fields of chemistry involving host–guest recognition.

At present, the route to new types of microporous frameworks depends on trial-and-error modification of existing templates or exhaustive searches for molecules with particular structural motifs conducive to the formation of the framework. (We have used the word 'template' to describe all molecules that possess structure-directing properties, but we note that the extent of the structure-directing property varies from molecule to molecule and from structure to structure.) Previous studies^{8–12} of the structure-directing effect have concentrated on the correlation between the van der Waals shape of the template and the resulting framework structure. Indeed, the van der Waals interactions can be used to determine the efficacy of the template at forming a particular framework¹¹—a quantification of the 'good fit' noted empirically^{8,9}. Although there are other properties of the organic species—especially the charge¹³, the hydrophobicity, the flexibility and the rotational dynamics—which will be vital for the successful synthesis of a material, the size and shape are clearly important starting points for any selection process, particularly in the case of three-dimensional channel systems.

De novo ligand design methods have recently been demonstrated to be useful in generating leads in the search for biologically active molecules^{14–19}. Growth is permitted, in a computational sense, to maximize favourable interactions between the substrate and an active site. The new code

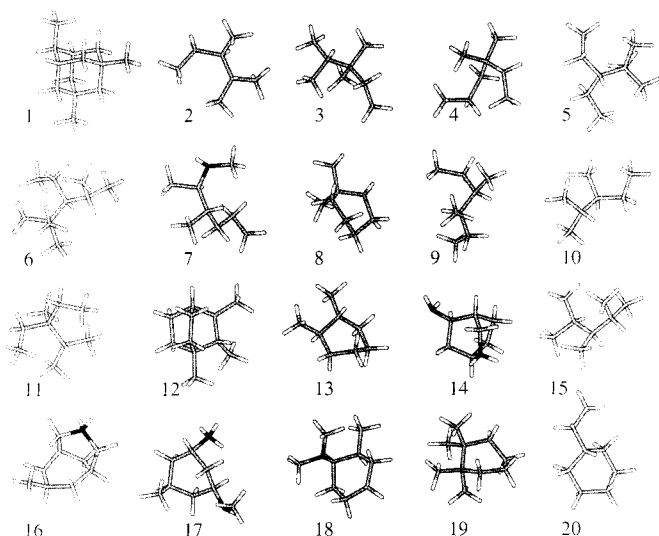


FIG. 1 A sample of 20 molecular structures produced (by the program ZEBEDDE) from methane as candidate templates for levyne, using a fragment library consisting of methane, ethane, propane, ammonia, ammonium, methylamine, cyclohexane, benzene, quinuclidine and adamantane. Note the tendency to form *cis*-isomers of alkanes and rings that mimic the shape of the zeolite. The former are unsuitable as templates owing to their high flexibility. However, our ring-formation procedure allows their conversion to the more rigid cyclic molecules. Close analogues of the experimentally utilized templates, 1-aminoadamantane and *N*-methylquinuclidinium are present (**1** and **12**, respectively), the former being constructed by the direct inclusion of an adamantyl fragment, the latter by the step-wise construction of the cyclic moiety. Black atoms are N, white are H and the remainder C.

ZEBEDDE (ZEolites By Evolutionary *De novo* DESIGN) has been written for the application of *de novo* techniques to microporous materials. We have included many features which are especially relevant in the synthesis of microporous solids, particularly the consideration of periodicity and symmetry of the growing molecule with respect to the host.

The target space (the host) in which a molecule (the template) is to be grown is supplied (as atomic coordinates) by the user, along with a library of fragments from which the template is to be constructed. Growth is initiated from a seed molecule placed (randomly or specifically) in the host. The molecule then grows by a number of random actions using the fragment library as the source of new atoms. The following actions are available. (1) *Build*, where a new (randomly selected) fragment is joined onto the existing template, by forming a bond between the neighbouring atoms of selected hydrogens, with these hydrogens being deleted from the molecule. Successful addition of a new fragment is controlled by a van der Waals overlap function and scaling the van der Waals radii is permitted to encourage growth. If overlap occurs, attempts are made using additional actions ((2)–(7) below), to reduce this conflict. Otherwise the addition is rejected. (2) *Rotate*, where the last fragment added is rotated about the new bond formed. (3) *Shake*, and (4) *Rock*, where the template is displaced along a random vector or randomly rotated with respect to the host, respectively. (5) *Random bond twist*, where a randomly selected bond joining fragments in the template is rotated. (6) *Ring formation*, where atoms which are *n*th-order neighbours and are within a cut-off distance are joined, forming a ring. (7) *Energy minimization*, where the template geometry is optimized either in the gas phase or with respect to the (fixed) host. Currently the code supports both Discover²⁰ and MOPAC²¹ as external minimizing programs.

Previous work¹¹ has shown that successful structure-directing agents, on the whole, must effectively fill the void space of the framework. Therefore, a cost function based on overlap of

van der Waals spheres is used to control the development of new templates:

$$f_c = \sum_i C(tz)/n \quad (1)$$

where $C(tz)$ signifies the closest contact between the template atom t and any host atom z and n is the number of atoms in the template. Therefore, to ensure that the molecule is effectively positioned within the available void space, the value of f_c must be maximized (that is, to facilitate growth, a molecule must be positioned as far as possible from the framework). But subsequently we should arrive at a position where further growth is no longer possible and now f_c provides a measure of the 'tightness of fit'. New growth is accepted if f_c has decreased and there are no geometric clashes. Simple application of this procedure, however, restricts growth in such confined voids; therefore a proportion (specified by the user) of actions are accepted without recourse to any evaluation. During actions (2)–(6), movements of the template are made as a series of small steps, and the action is repeated if f_c increases. Such a strategy allows the template to locate rapidly the largest available space in the host, and to achieve there its optimum geometric complementarity with the host. We note that our approach contrasts with that used in pharmaceutical applications^{13–19} where growth is biased towards maximizing specific host–guest interactions. Growth is terminated when the cost function attains a pre-ordained value, or when all the replaceable hydrogens are exhausted, or when a pre-set number of actions has been performed. The actions and fragments can be weighted by the user, as can the relative weightings of the replaceable hydrogens. The latter can be used to bias for example, linear, branched or cyclic molecules.

Recognizing the crystalline nature of microporous materials, we have implemented periodic boundary conditions. Furthermore, the symmetry of the template may be specified so that the concentration of the template may, if need be, be higher than one per unit cell. It therefore follows that a number of symmetry-related templates may be grown in the unit cell. Thus a growing template interacts with both its periodic and symmetry images.

To demonstrate the potential of this technique in developing new templates we have performed a number of simulations on different types of zeolitic topologies. We consider first, the zeolite mineral levyne, which possesses a cage structure. By including cyclic fragments in our library, simulations resulted in one-step growth toward molecules which are very similar to those experimentally employed²² (for example, **1** and **12**) (Fig. 1). (Although the cyclic fragments are included in our library, we stress that the

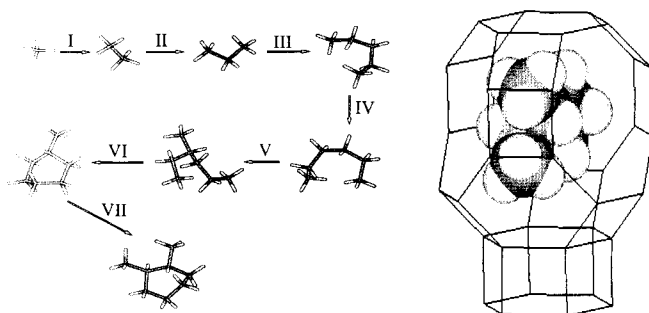


FIG. 2 Left, the growth processes involved in the formation of the di-substituted cyclohexane derivative (template **13** in Fig. 1) in the levyne structure. Starting from a methane seed, the alkyl chain grows inside the restricted void of the levyne structure (I–V). High bias of the ring formation action (see text) results in the formation of methyl-cyclohexane in step VI, when an atom with a fifth-order neighbour within a distance of 3 Å is selected. Further substitution of this ring then occurs. Right, the calculated position of the template in the levyne cage, the cage being represented by the positions of the cations in the framework, with the bridging oxygen, situated approximately halfway between the cation vertices, being omitted for clarity.

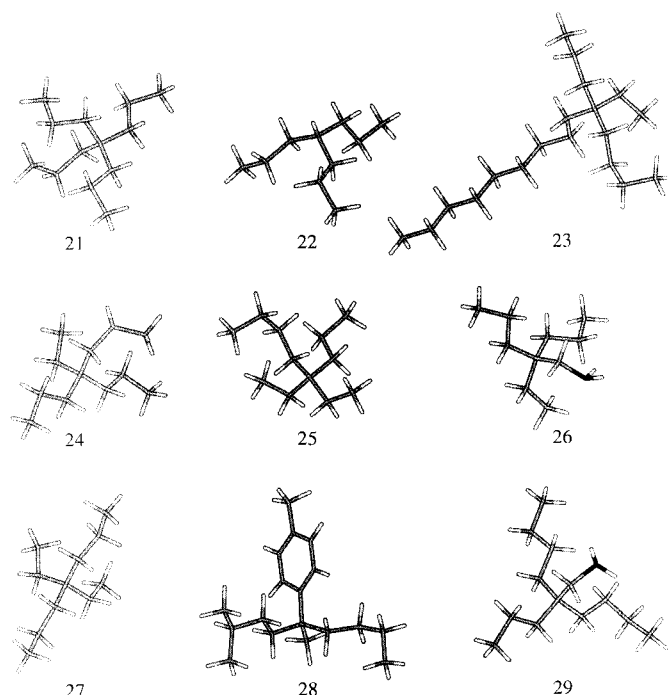


FIG. 3 A sample of candidate templates grown in the ZSM-5 structure. Molecules **21–25** are examples grown in a non-periodic supercell of the ZSM-5 structure from an initial methane seed, using the same fragment library as in the simulations of levyne. Of those three-dimensional templates grown, alkyl analogues of both tetrapropylammonium (**21**) and tripropylammonium (**22**) are present. The specification of periodic boundary conditions and the imposition of template symmetry (requiring growth of four molecules per P1 unit cell) generates structures such as **21**, **22**, **26–29**. Note how long branches are reflected by short branches due to the periodicity (for example, **27** and **28**). Owing to the reduced probability of a replaceable hydrogen being randomly selected, tri-substituted templates (such as **22**) dominate unless selection of hydrogens is biased towards those which have been present in the growing molecules longer—quater-nized (**21**, **26–29**) molecules then become prevalent.

code automatically generated the template from methane, demonstrating the effectiveness of our growth control.) The simulation population was dominated by branched chain alkanes and aminoalkanes (for example, **2**, **11**), which would be unsuitable as templates due to their high flexibility. But by encouraging ring formation, these molecules convert to more rigid cyclic species (for example, **13–20**). Of particular note is the growth of the templates containing the bridged-ring quinuclidine motif (for example, **12**) using only methyl and ethyl fragments.

More significantly, the process of ring-formation resulted in the production of the di-substituted cyclohexane derivative, 1,2-dimethylcyclohexane (**13**) as a possible candidate, with a high binding energy (Fig. 2). Other workers in our laboratory independently used the amino analogue of this template, 2-methylcyclohexylamine, to form a microporous cobalt-aluminophosphate solid acid that crystallizes with the levyne structure, designated DAF-4 (ref. 23).

Next, we considered the ZSM-5 structure, which possesses intersecting channels. Using a finite supercell of ZSM-5 we generated a number of linear alkanes and amines with carbon chains varying in length from 4 to 8, located primarily away from the channel intersection, similar to the range of experimentally proven α,ω -diaminoalkanes and n -amines. We also found a number of candidates (for example, **21–23**; Fig. 3) which match the three-dimensional shape of the channel intersection including

alkyl analogues of tetrapropylammonium (**21**) and tripropylamine (**22**). Neglect of the periodicity of the template however, allows the molecules to continue growing unhindered along the channels, as for **28**. These results suggest that, in general, a periodic unit cell should be used and that specification of template symmetry is important. We therefore specified the template symmetry such that one molecule would grow per intersection (four per unit cell), as found for tetrapropylammonium²⁴, although we stress that the code itself finds the intersection to be the optimal site from the initial random position. Analogues of experimentally used templates were again present in this sample (**21** and **22**). Furthermore, it is clear (Fig. 3) how the interactions with their symmetry images further controls the growth of the molecules.

Although the code described, ZEBEDDE, was primarily developed for use with microporous materials, we have developed it in a general manner so as to be applicable to other fields of chemistry such as the development of pharmaceuticals. Our method clearly has great scope for generating new templates for existing materials. Furthermore, because there are an infinite number of hypothetical 'zeolitic' structures and there are only small thermodynamic limitations for the formation of framework structures²⁵, there is considerable potential for this method in producing candidate templates for the synthesis of new microporous materials. □

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1. Barrer, R. M. *Hydrothermal Chemistry of Zeolites* (Academic, London, 1992).
2. Davis, M. E. *Chemtech* **24**, 22–26 (1994).
3. Zones, S. I. & Santilli, D. S. in *9th Int. Zeolites Conf.* (eds von Ballmoos, R. et al.) 171–179 (Butterworth-Heinemann, Montreal, 1992).
4. Wright, P. A. et al. *J. chem. Soc. chem. Commun.* 633–635 (1993).
5. Chen, J. & Thomas, J. M. *J. chem. Soc. chem. Commun.* 603–604 (1994).
6. Schmitt, K. D. & Kennedy, G. J. *Zeolites* **14**, 635–642 (1994).
7. Lobo, R. F. & Davis, M. E. *J. Am. chem. Soc.* **117**, 3766–3779 (1995).
8. Gies, H. & Marler, B. *Zeolites* **12**, 42–49 (1992).
9. Gies, H. *Stud. Surf. Sci. Catal.* **85**, 295–327 (1994).
10. Bell, R. G. et al. in *Proc. 10th Int. Zeolite Ass. Meeting* (eds Weitkamp, J., Karge, H. G., Pfeifer, H. & Holderich, W.) 2075–2087 (Elsevier, Amsterdam, 1994).
11. Lewis, D. W., Freeman, C. M. & Catlow, C. R. A. *J. phys. Chem.* **99**, 11194–11202 (1995).
12. Cox, P. A., Stevens, A. P., Banting, L. & Gorman, A. M. in *Proc. 10th Int. Zeolite Ass. Meeting* (eds Weitkamp, J., Karge, H. G., Pfeifer, H. & Holderich, W.) 2115–2122 (Elsevier, Amsterdam, 1994).
13. Lewis, D. W., Catlow, C. R. A. & Thomas, J. M. *Chem. Mater.* **8**, 1112–1121 (1996).
14. Bohacek, R. S. & McMartin, C. J. *J. Am. chem. Soc.* **116**, 5560–5571 (1994).
15. Bohm, H. J. *J. Comput.-Aided Molec. Design* **6**, 61–78 (1992).

16. Gillet, V., Johnson, A. P., Mata, P., Sike, S. & Williams, P. J. *J. Comput.-Aided Molec. Design* **7**, 127–153 (1993).
17. Lewis, R. A. & Dean, P. M. *Proc. R. Soc. Lond. B* **236**, 125–140 (1989).
18. Oprea, T. I., Ho, C. M. W. & Marshall, G. R. in *Computer-Aided Molecular Design. Applications in Agrochemicals, Materials and Pharmaceuticals* (eds Reynolds, C. H., Holloway, M. K. & Cox, H. K.) 65–81 (American Chemical Soc., Washington DC, 1995).
19. Verlinde, C., Rudenko, G. & Hol, W. J. *Comput.-Aided Molec. Design* **6**, 131–147 (1992).
20. Discover 2.9.7 (Molecular Simulations Inc. San Diego, 1995).
21. Stewart, J. J. P. *Quantum Chemical Program Exch. Bull.* **3**, 24 (1983).
22. McCusker, L. B. *Mater. Sci. Forum* **133–136**, 423–434 (1993).
23. Barratt, P. A. et al. *J. chem. Soc. chem. Commun.* (in the press).
24. Price, G. D., Pluth, J. J., Smith, J. V., Araki, T. & Bennett, J. M. *Nature* **292**, 818–819 (1981).
25. Navrotsky, A., Petrovic, I., Hu, Y. T., Chen, C. Y. & Davis, M. E. *Microporous Mater.* **4**, 95–98 (1995).

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CORRESPONDENCE should be addressed to D.W.L. (e-mail: dewi@ri.ac.uk).

A DNA-based method for rationally assembling nanoparticles into macroscopic materials

Chad A. Mirkin, Robert L. Letsinger, Robert C. Mucic & James J. Storhoff

Department of Chemistry, Northwestern University, Evanston, Illinois 60208, USA

COLLOIDAL particles of metals and semiconductors have potentially useful optical, optoelectronic and material properties¹⁻⁴ that derive from their small (nanoscopic) size. These properties might lead to applications including chemical sensors, spectroscopic enhancers, quantum dot and nanostructure fabrication, and microimaging methods²⁻⁴. A great deal of control can now be exercised over the chemical composition, size and polydispersity^{1,2} of colloidal particles, and many methods have been developed for assembling them into useful aggregates and materials. Here we describe a method for assembling colloidal gold nanoparticles rationally and reversibly into macroscopic aggregates. The method involves attaching to the surfaces of two batches of 13-nm gold particles non-complementary DNA oligonucleotides capped with thiol groups, which bind to gold. When we add to the solution an oligonucleotide duplex with 'sticky ends' that are complementary to the two grafted sequences, the nanoparticles self-assemble into aggregates. This assembly process can be reversed by thermal denaturation. This strategy should now make it possible to tailor the optical, electronic and structural properties of the colloidal aggregates by using the specificity of DNA interactions to direct the interactions between particles of different size and composition.

Previous assembly methods have focused on the use of covalent 'linker' molecules that possess functionalities at opposing ends with chemical affinities for the colloids of interest. One of the most successful approaches to date⁵ has involved the use of gold colloids and well established thiol adsorption chemistry^{6,7}. In this approach, linear alkanedithiols were used as the particle linker molecules. The thiol groups at each end of the linker molecule covalently attach themselves to the colloidal particles to form aggregate structures. The drawbacks of this method are that the process is difficult to control and the assemblies are formed irreversibly. Methods for systematically controlling the assembly process are needed if the materials properties of these unusual structures are to be exploited fully.

Our oligonucleotide-based method allows the controlled and reversible assembly of gold nanoparticles into supramolecular structures. Oligonucleotides offer several advantages over non-biological-based linker molecules. For example, discrete sequences of controlled length and with the appropriate surface binding functionality may be prepared in an automated fashion with a DNA synthesizer. In this way, the molecular recognition properties of the oligonucleotides may be used to trigger the colloidal self-assembly process. The interparticle distances and stabilities of the supramolecular structures generated by this method can be controlled through the choice of oligonucleotide sequence and length, solvent, temperature and supporting electrolyte concentration.

Others also have recognized the utility of DNA for the preparation of new biomaterials and nanofabrication methods. Previous researchers have focused on using the sequence-specific molecular-recognition properties of oligonucleotides to design impressive structures with well defined geometric shapes and sizes⁸⁻¹⁸. The chemistry proposed here focuses on merging the chemistry of DNA with the chemistry of inorganic colloidal

materials. In addition to generating materials with properties that are hybrids of their DNA and colloidal precursors, the union of metal-colloid and DNA chemistry offers significant opportunities relative to the construction of pure DNA materials. As noted by Seeman¹⁹, 'the theory of producing DNA [structures] is well ahead of experimental confirmation. It is much easier to design a [structure] than it is to prove its synthesis.' An advantage of the DNA/colloid hybrid materials reported herein is that the assemblies can be characterized easily by transmission electron microscopy (TEM) and/or atomic force microscopy (AFM) as well as spectroscopic methods conventionally used with DNA.

Our approach to using oligonucleotides for the controlled assembly of gold nanoparticles into aggregate macroscopic structures is outlined in Fig. 1. First, 13-nm-diameter Au particles are prepared^{2,20}. These particles form a dark red suspension in water, and like thin-film Au substrates²¹, they are easily modified with oligonucleotides, which are functionalized with alkane thiols at their 3' termini. In a typical experiment, one solution of 17 nM (150 μ l) Au colloids is treated for 24 h with 3.75 μ M (46 μ l) 3'-thiol-TTTGCTGA, and a second solution of colloids is treated with 3.75 μ M (46 μ l) 3'-thiol-TACCGTTG. Note that these oligonucleotides are non-complementary. After treatment with the thiol-capped oligonucleotides, the two colloidal Au solutions are combined, and because of the non-complementary nature of the oligonucleotides, no reaction takes place. A beneficial consequence of capping the colloids with these oligonucleotides is

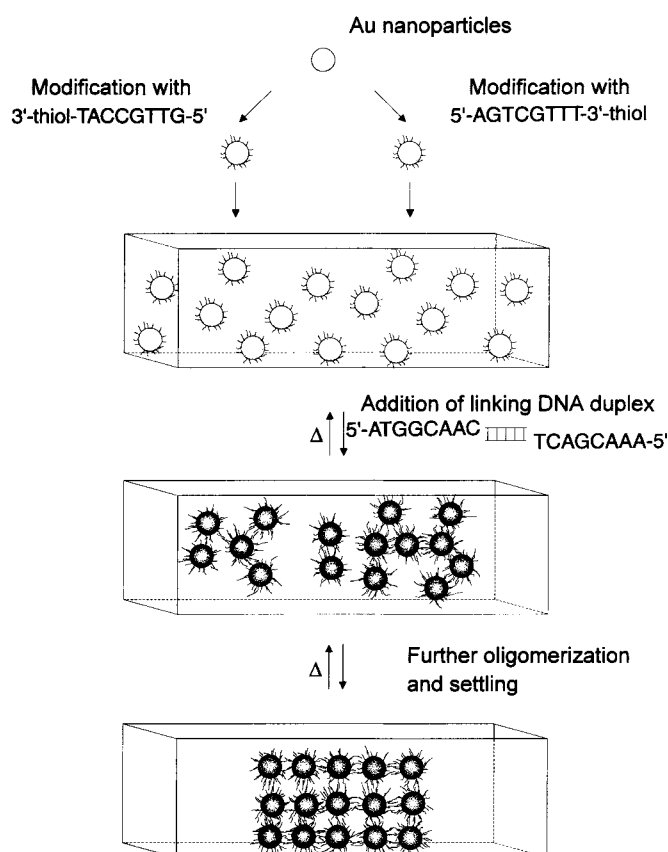


FIG. 1 Scheme showing the DNA-based colloidal nanoparticle assembly strategy (the hybridized 12-base-pair portion of the linking duplex is abbreviated as $\square\square\square$). If a duplex with a 12-base-pair overlap but with 'sticky ends' with four base mismatches (5'-AAGTCAGTTATACGCGCTAG and 3'-ATATGCGCGATCAAATCACA) is used in the second step, no reversible particle aggregation is observed. The scheme is not meant to imply the formation of a crystalline lattice but rather an aggregate structure that can be reversibly annealed. Δ is the heating above the dissociation temperature of the duplex.



FIG. 2 Cuvettes with the Au colloids and the four DNA strands responsible for the assembly process. Left cuvette, at 80 °C with DNA-modified colloids in the unhybridized state; centre, after cooling to room temperature but before the precipitate settles; and right, after the polymeric precipitate settles to the bottom of the cuvette. Heating either of these cool solutions results in the reformation of the DNA-modified colloids in the unhybridized state (shown in the left cuvette).

that they are much more stable than bare Au colloids to increased salt concentration and temperature. When heated or in a solution of high salt concentration (0.1 M NaCl), bare colloids undergo irreversible particle-growth reactions that result in their precipitation. In contrast, the DNA-modified Au nanoparticles reported here are stable at elevated temperatures (80 °C) and in aqueous 0.1 M NaCl solutions for days, presumably because their DNA-modified surfaces prohibit them from getting close enough to undergo particle growth. This is important because high salt concentrations are needed for the DNA hybridization events depicted in Fig. 1.

In the next step of the assembly scheme, a duplex consisting of 5'-ATGGCAACTATACGCGCTAG and 3'-ATATGCGCGA-TCTCAGCAAA (the duplex has a 12-base-pair overlap (underlined), containing 8-base-pair sticky ends, which are complementary to the 8-base-pair oligonucleotides that are covalently attached to the Au colloids; Fig. 1) is added to the dark red solution. The solution is then diluted with aqueous NaCl (to 1 M) and buffered at pH 7 with 10 mM phosphate, conditions which are suitable for hybridization of the oligonucleotides. Significantly, an immediate colour change from red to purple is observed and a precipitation reaction ensues. Over the course of several hours, the solution becomes clear and a pinkish-grey precipitate settles to the bottom of the reaction vessel (Fig. 2). Presumably, the free ends of the

'linking' duplex bind to the complementary oligomers anchored to the gold, thereby crosslinking the colloids, which ultimately results in the formation of the pinkish-grey polymeric DNA-colloid precipitate. To verify that this process involved both the DNA and colloids, the precipitate was collected and resuspended (by shaking) in 1 M aqueous NaCl buffered at pH 7. Then, a temperature/time dissociation experiment was performed by monitoring both an optical absorption dependent on hybridization of DNA (260 nm) and one dependent on the degree of colloid aggregation (700 nm), Fig. 3a. As the temperature is cycled between 0 and 80 °C, which is 38 °C above the dissociation temperature (T_m) for the DNA-duplex ($T_m = 42$ °C), there is an excellent correlation between the optical signatures for both the colloids and DNA. In the absence of DNA, the ultraviolet-visible spectrum for the naked Au colloids is much less temperature-dependent (Fig. 3b). There is a substantial optical change when the polymeric DNA-colloid precipitate is heated above its melting point. The clear solution turns dark red as the polymeric biomaterial dehybridizes to generate the unlinked colloids which are soluble in the aqueous solution. This process is very reversible as evidenced by the temperature traces in Fig. 3a. In a control experiment designed to verify that this process was due to oligonucleotide hybridization, a duplex with four base-pair mismatches in each of the 'sticky' ends of the linkers (step 2 in Fig. 1) did not induce the reversible particle aggregation process.

Further evidence of the polymerization/assembly process comes from TEM studies of the polymeric precipitate (Fig. 4). TEM images of the colloids linked with hybridized DNA show large assembled networks of the Au colloids (Fig. 4a). Naked Au colloids do not aggregate in this manner under comparable conditions, but rather undergo particle-growth reactions². Note that there is no evidence of colloid particle growth as the hybridized colloids seem to be remarkably regular in size with an average diameter of 13 nm. With TEM, because of the superposition of layers, it is difficult to assess the degree of order for three-dimensional aggregates. But smaller-scale images of single-layer, two-dimensional aggregates provide more compelling evidence of the self-assembly process (Fig. 4b). This figure shows close-packed assemblies of the aggregates with uniform particle separations ~ 60 Å. This distance is somewhat shorter than the maximum spacing (95 Å) expected for colloids connected by rigid DNA hybrids with the selected sequences. But because of the nicks in the DNA duplex, these are not rigid hybrids and are quite flexible. It should be noted that, in principle, this is a variable that can be controlled by reducing the system from four overlapping strands to three (thereby reducing the number of nicks) or by using triplexes instead of duplexes.

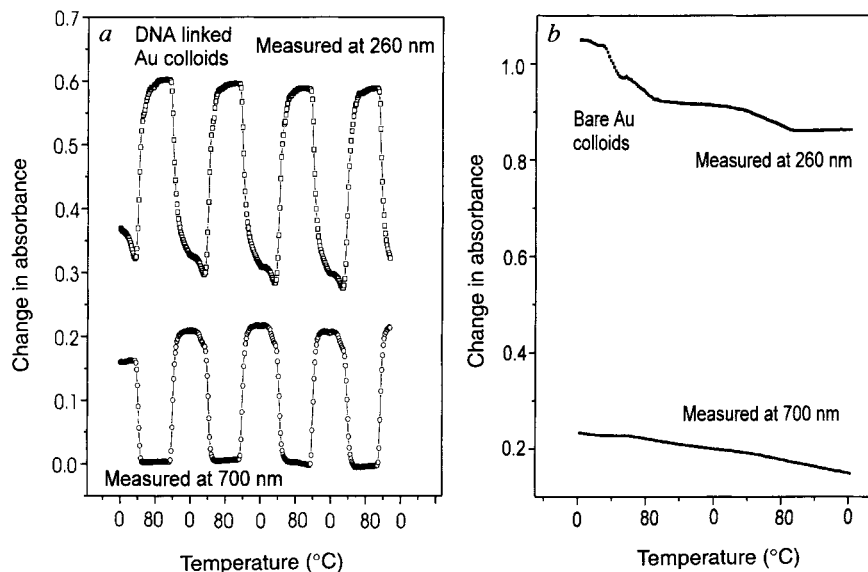


FIG. 3 a, Absorbance versus temperature/time profile for DNA/colloid hybridized materials. At low temperatures the Au colloids aggregate owing to the hybridization of 'linking' DNA. At high temperature (80 °C), the colloids dehybridize and form a dark red solution (see Fig. 1 and Fig. 2). The temperature versus time profile shows that this is a reversible process. b, Results of same procedure shown in a, but applied to an aqueous solution of unmodified Au colloids (5.1 nM, same concentration as in a).

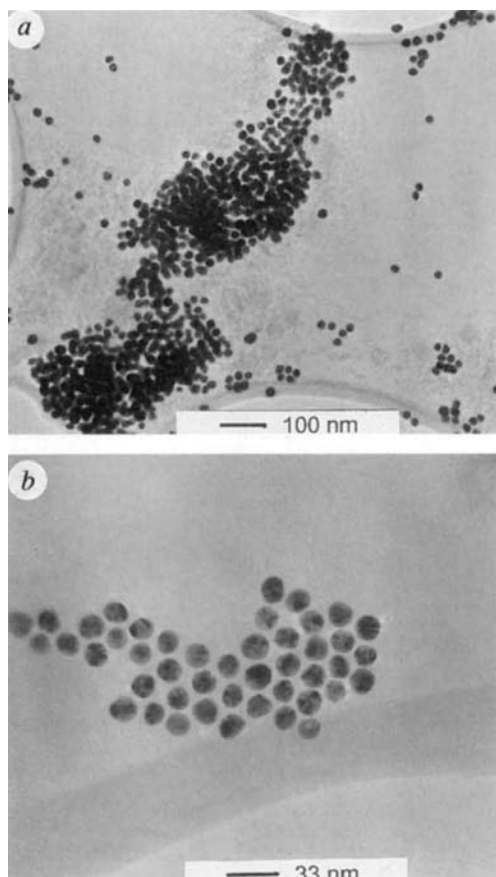


Fig. 4 TEM images of: a, an aggregated DNA/colloid hybrid material; b, a two-dimensional colloidal aggregate showing the ordering of the DNA-linked Au nanoparticles. Images were taken with a Hitachi 8100 Transmission Electron Microscope.

This work gives entry into a new class of DNA/nanoparticle hybrid materials and assemblies, which might have useful electrical, optical and structural properties that should be controllable through choice of nanoparticle size and chemical composition, and oligonucleotide sequence and length. We note that it should be possible to extend this strategy easily to other noble-metal (for example, Ag, Pt)²² and semiconductor (for example, CdSe and CdS)^{23,24} colloidal nanoparticles with well established surface coordination chemistry. Our initial results bode well for the utility of this strategy for developing new types of biosensing and sequencing schemes for DNA. The Au colloidal particles have large extinction coefficients for the bands that give rise to their colours (Fig. 2). These intense colours, which depend on particle size and concentration and interparticle distance, make these materials particularly attractive for new colorimetric sensing and sequencing strategies for DNA. □

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- Schmid, G. (ed.) *Clusters and Colloids* (VCH, Weinheim, 1994).
- Hayat, M. A. (ed.) *Colloidal Gold: Principles, Methods, and Applications* (Academic, San Diego, 1991).
- Bassell, G. J., Powers, C. M., Taneja, K. L. & Singer, R. H. *J. Cell Biol.* **126**, 863–876 (1994).
- Creighton, J. A., Blatchford, C. G. & Albrecht, M. G. *J. chem. Soc. Faraday II* **75**, 790–798 (1979).
- Brust, M., Bethell, D., Schiffrin, D. J. & Kiely, C. J. *Adv. Mater.* **7**, 795–797 (1995).
- Dubois, L. H. & Nuzzo, R. G. *Rev. phys. Chem.* **43**, 437–463 (1992).
- Bain, C. D. & Whitesides, G. M. *Angew. Chem. int. Edn. engl.* **28**, 506–512 (1989).
- Shekhtman, E. M., Wasserman, S. A., Cozzarelli, N. R. & Solomon, M. J. *New J. Chem.* **17**, 757–763 (1993).
- Shaw, S. Y. & Wang, J. C. *Science* **260**, 533–536 (1993).
- Herrlein, M. K., Nelson, J. S. & Letsinger, R. L. *J. Am. Chem. Soc.* **117**, 10151–10152 (1995).
- Chen, J. H. & Seeman, N. C. *Nature* **350**, 631–633 (1991).
- Smith, F. W. & Feigon, J. *Nature* **356**, 164–168 (1992).
- Wang, K. Y., McCurdy, S., Shea, R. G., Swaminathan, S. & Bolton, P. H. *Biochemistry* **32**,

1899–1904 (1993).

- Chen, L. Q., Cai, L., Zhang, X. H. & Rich, A. *Biochemistry* **33**, 13540–13546 (1994).
- Marsh, T. C., Veselka, J. & Henderson, E. *Nucleic Acids Res.* **23**, 696–700 (1995).
- Mirkin, S. M. & Frankkamenetskii, M. D. A. *Rev. Biophys. biomolec. Struct.* **23**, 541–576 (1994).
- Wells, R. D. *J. biol. Chem.* **263**, 1095–1098 (1988).
- Wang, Y., Mueller, J. E., Kemper, B. & Seeman, N. C. *Biochemistry* **30**, 5667–5674 (1991).
- Seeman, N. C. et al. *New J. Chem.* **17**, 739–755 (1993).
- Grabar, K. C., Freeman, R. G., Hommer, M. B. & Natan, M. J. *Analyt. Chem.* **67**, 735–743 (1995).
- Mucic, R. C., Herrlein, M. K., Mirkin, C. A. & Letsinger, R. L. *J. chem. Soc., chem. Commun.* 555–557 (1996).
- Linnert, T., Mulvaney, P. & Henglein, A. *J. phys. Chem.* **97**, 679–682 (1993).
- Herron, N., Wang, Y. & Eckert, H. J. *Am. chem. Soc.* **112**, 1322–1326 (1990).
- Colvin, V. L., Goldstein, A. N. & Alivisatos, A. P. *J. Am. chem. Soc.* **114**, 5221–5230 (1992).

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CORRESPONDENCE should be addressed to C.A.M. (e-mail: camirkin@chem.nwu.edu).

Organization of 'nanocrystal molecules' using DNA

A. Paul Alivisatos*, Kai P. Johnsson†, Xiaogang Peng*, Troy E. Wilson†, Colin J. Loweth†, Marcel P. Bruchez Jr* & Peter G. Schultz†

* Department of Chemistry, University of California at Berkeley, Berkeley, California 94720, USA and Molecular Design Institute, Lawrence Berkeley National Laboratory, Berkeley, California 94701, USA

† Howard Hughes Medical Institute, Department of Chemistry, University of California at Berkeley, Berkeley, California 94720, USA

PATTERNING matter on the nanometre scale is an important objective of current materials chemistry and physics. It is driven by both the need to further miniaturize electronic components and the fact that at the nanometre scale, materials properties are strongly size-dependent and thus can be tuned sensitively¹. In nanoscale crystals, quantum size effects and the large number of surface atoms influence the, chemical, electronic, magnetic and optical behaviour^{2–4}. 'Top-down' (for example, lithographic) methods for nanoscale manipulation reach only to the upper end of the nanometre regime⁵; but whereas 'bottom-up' wet chemical techniques allow for the preparation of monodisperse, defect-free crystallites just 1–10 nm in size^{6–10}, ways to control the structure of nanocrystal assemblies are scarce. Here we describe a strategy for the synthesis of 'nanocrystal molecules', in which discrete numbers of gold nanocrystals are organized into spatially defined structures based on Watson-Crick base-pairing interactions. We attach single-stranded DNA oligonucleotides of defined length and sequence to individual nanocrystals, and these assemble into dimers and trimers on addition of a complementary single-stranded DNA template. We anticipate that this approach should allow the construction of more complex two- and three-dimensional assemblies.

Previous approaches towards the preparation of coupled quantum dots include co-colloids of cadmium selenide–zinc oxide (CdS–ZnO; ref. 11) and cadmium sulphide–silver iodide (CdS–AgI; ref. 12). In addition, small molecule crosslinking agents have been used to synthesize aggregates of Au (ref. 13) and cadmium sulphide linked to titanium oxide (CdS–TiO₂; ref. 14) as well as discrete dimers of cadmium selenide (CdSe; ref. 15). Finally, the collective properties of nanocrystals have been investigated using organic monolayers^{16–22} and crystallization^{23–26} to generate ordered arrays of inorganic quantum dots. It remains an open question whether self-assembly methods can be employed to generate complex sequences of nanocrystals.

Biological systems are characterized by remarkably complex

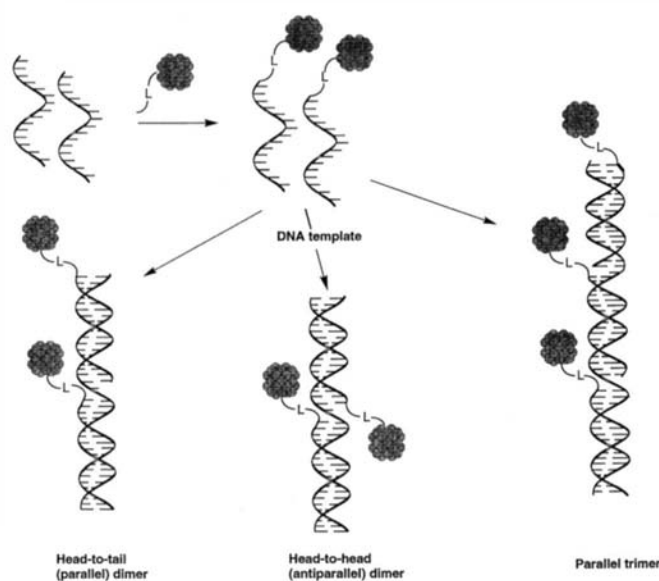


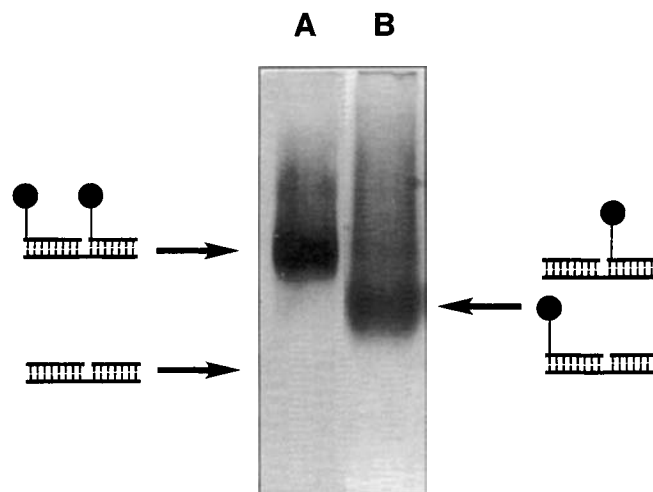
FIG. 1 Nanocrystal assembly based on Watson-Crick base-pairing interactions. Attachment of the inorganic nanocrystal (shaded) to either the 3' or 5' termini of the oligonucleotide 'codon' by linker L, permits the preparation of head-to-head dimers, head-to-tail dimers, and trimers, as shown schematically here and observed in the TEM images of Fig. 3. METHODS. The 'codon' and template oligonucleotides were prepared using an Applied Biosystems Model 391 DNA Synthesizer. Sulphydryl groups were introduced at the 5' or 3' termini of 18-nucleotide (nt) synthetic oligonucleotides by capping with S-trityl-6-mercaptopropylphosphoramidite or by using 1-O-dimethoxytritylpropyldisulphide-1'-succinoyl support (Glen

Research, Sterling, Virginia), respectively. The 5'-thiol group was detritylated using silver nitrate and dithiothreitol following the procedure of Zuckermann *et al.*²⁹. Oligonucleotides thiolated at the 3' terminus were oxidized using a 0.02 M iodine solution after each coupling step and cleaved from support with 0.05 M dithiothreitol during deprotection in concentrated NH_4OH . All oligonucleotides were purified by gel electrophoresis or reverse-phase high-performance liquid chromatography (HPLC). Concentrations were determined spectrophotometrically and by titration of free thiol using 5,5'-dithiobis(2-nitrobenzoic) acid. Thiol-modified oligonucleotides were stored at -20°C in aqueous 50 mM dithiothreitol. Oligonucleotides were desalted on a Pharmacia Superdex 75 HR 10/30 column using 3 mM bis-Tris, 1 mM EDTA buffer, pH 6.5. Extinction coefficients were determined by combining standard extinction coefficients for the deoxynucleotide triphosphates (dNTPs). Sequences prepared for these experiments include: the 5'-thiol-terminated 18-nt oligomer (5'-HS-CAGTCAGGCAGTCAGTCA-3') (oligo **1**); the 37-nt template (5'-TGACTGACTGCCTGACTGTTGACTGACTGCCTGACTGTTGACTGACTGCCTGACTG-3') (template **2**); the 5'-thiol-terminated 18-nt oligomer (5'-HS-CTTGCACTAGTCCTTGAG-3') (oligo **3**); the 3'-thiol-terminated 18-nt oligomer (5'-CAGTCAGGCAGTCAGTCA-SH-3') (oligo **4**); the 37-nt template (5'-CTCAAGGACTAGTGCAAGTTGACTGACTGCCTGACTGTTGACTGACTGCCTGACTGTTGACTGACTGCCTGACTG-3') (template **6**); and the unlabelled 18-nt oligomer (5'-CAGTCAGGCAGTCAGTCA-3') (oligo **7**). Oligonucleotides, modified at either the 3' or 5' termini with a free sulphydryl group ($1.5\ \mu\text{M}$) were coupled with a 10-fold excess of the monomaleimido-Au particles (Nanoprobe, Stony Brook, NY) in aqueous 20 mM NaH_2PO_4 , 150 mM NaCl, 1 mM EDTA buffer, pH 6.5, containing 10% isopropanol at 4°C for 24 h. The oligonucleotide-Au conjugates were then combined with the appropriate amounts of 37-nt single-stranded oligonucleotide templates and the resulting double-stranded complexes were purified from single-stranded precursors on a sizing column (Superose 12 10/30 in aqueous 5 mM NaH_2PO_4 , 150 mM NaCl buffer, pH 6.5). The ratio of absorbances (A_{280}/A_{420}), determined spectrophotometrically, indicated that the complex had the desired stoichiometries of oligonucleotide and Au cluster.

To test the feasibility of this approach, we chose as initial targets

the head-to-head (antiparallel) and head-to-tail (parallel) homo-dimers shown in Fig. 1. Gold (Au) clusters, 1.4 nm in diameter and passivated with water-soluble phosphine ligands (Nanoprobe, Stony Brook, New York), were used for these investigations. The particles contain one *N*-propylmaleimide substituent per cluster which can be selectively coupled to a sulphydryl group incorporated into the single-stranded DNA 'codon'. Oligonucleotides, modified at either the 3' or 5' termini with a free sulphydryl group, were coupled with an excess of monomaleimido-Au particles. The oligonucleotide-Au conjugates were then combined with appropriate amounts of 37-nucleotide single-stranded templates and the resulting double-stranded complexes were purified from single-stranded precursors on a sizing column. The retention times of the DNA-Au conjugates were significantly different from the retention times of the corresponding Au-oligonucleotide duplexes. The ratio of absorbances at

FIG. 2 Gel electrophoresis (10% acrylamide) results of DNA-Au complexes after purification by a sizing column; lane A, a 2:1 mixture of Au-labelled oligo **1** and the corresponding template **2**; lane B, a 1:1 mixture of Au-labelled oligo **1** and oligo **7** incubated with template **2**. The arrows indicate the retention times of the nucleic acid complexes containing two, one and zero Au clusters (indicated by the filled circles). Gel electrophoresis was accomplished using 10% crosslinked polyacrylamide gels³⁰ in $1\times$ TBE buffer at 80 mV. The Au-labelled oligonucleotides were visualized by development with Li Silver stain (Nanoprobe, Stony Brook, NY), and the non-Au-labelled duplexes were visualized by treatment with ethidium bromide. The band representing the template **2** annealed to two oligo **7** fragments was visualized using ethidium bromide staining but not with the Li Silver stain.



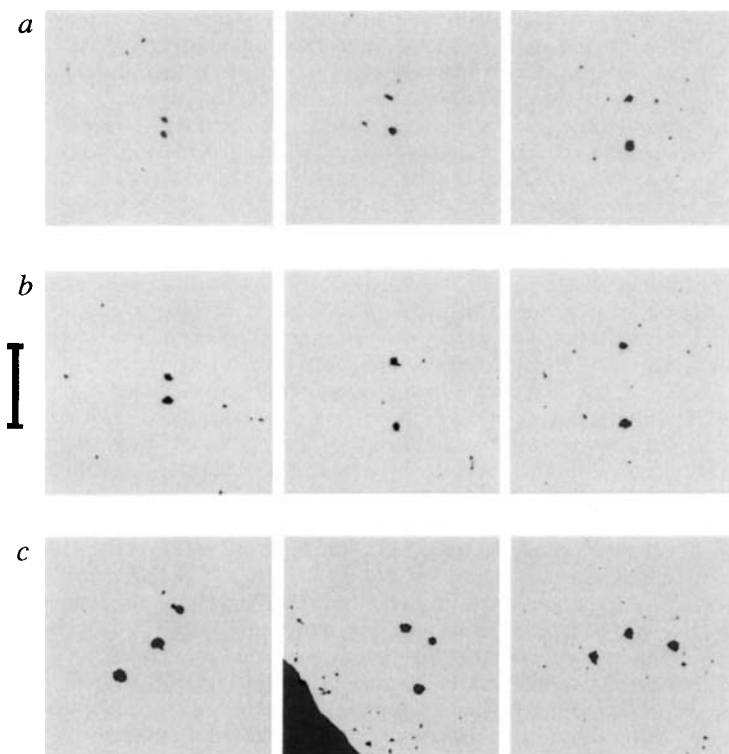


FIG. 3 Transmission electron microscopy (TEM) images of 1.4 nm Au-nucleic acid complexes. *a*, Head-to-head dimer sample in which a 2:1 ratio of oligo **1** was hybridized to the corresponding template **2**. From left to right are examples of the nearest, average and farthest dimers. The centre-to-centre distances range from 2.9 ± 0.6 to 10.2 ± 0.6 nm. *b*, Head-to-tail dimer sample in which a 1:1 ratio of oligo **3** and oligo **4** were hybridized to template **5**. From left to right are examples of the nearest, average and farthest dimers. The centre-to-centre distances in this example ranged from 2.0 ± 0.6 to 6.3 ± 0.6 nm. *c*, Equidistant trimer sample in which oligo **1** was hybridized to the trimeric template **6**. The curvature apparent in the trimer samples may reflect the flexibility of the linkers between the Au centres and the nucleic acids or bending in the DNA. Scale bar, 10 nm. The absorbances of Au-DNA samples used for imaging was approximately 5 mAU (where AU is absorbance units). A Jeol-100CX TEM instrument, operating at 80 kV, was used to image the samples which had been deposited on 400-mesh copper grids coated with ultra-thin (2–3 nm) holey carbon films. The carbon-coated grids were subjected briefly to an air plasma (30 seconds at 50 mtorr) followed by deposition of 10–15 μ l of a 10 mg per ml solution of polylysine (reactive molecular mass 10–40,000, pH 7.5; Sigma Chemical, St Louis, Missouri). The grids were dried in air, solutions of the DNA-Au complexes were deposited and the excess solution was removed by wicking. Examination of a large number of nanocrystal-DNA complexes by TEM indicated that ~70% were consistent with the expected dimeric structures.

280 nm to that at 420 nm (A_{280}/A_{420}), determined, indicated that the complex had spectrophotometrically the desired stoichiometries of oligonucleotide and Au cluster. The complexes were also analysed by gel electrophoresis using ethidium bromide and silver staining to visualize the oligonucleotides and oligonucleotide-Au conjugates, respectively (Fig. 2). In the case of the dimeric complexes, individual bands were observed that were assigned to double-stranded complexes containing two, one or zero Au particles. These assignments were confirmed by competing the oligonucleotide-Au conjugate with free oligomer and analysing the pattern and intensities of the resulting bands (Fig. 2).

Transmission electron microscopy (TEM) was used to characterize the DNA-Au nanocrystal molecules. Dilute samples (20–30 nM) were imaged on ultra-thin carbon films coated with polylysine solution (10 mg per ml) to enhance binding of the oligonucleotides. Experiments using a double-stranded oligonucleotide-Au control showed that 90% of the Au particles are well separated, thereby excluding the possibility of aggregation due to TEM sample preparation. The TEM images (Fig. 3*a, b*) of the DNA-Au complexes were consistent with the expected

dimeric structures. Modelling of the parallel and antiparallel dimers indicates that the centre-to-centre distances between the two Au particles can vary from 2.6 to 12.5 nm and 2.6 to 7.5 nm, respectively, due to the combined lengths of the linkers and the ligand shell. The distances observed under the TEM for the parallel and antiparallel dimers range from 2.9 ± 0.6 to 10.2 ± 0.6 nm and 2.0 ± 0.6 to 6.3 ± 0.6 nm, respectively. As additional evidence for our ability to chemically control nanocrystal molecules, we synthesized a trimeric parallel complex using a 56-nucleotide single-stranded DNA template (Fig. 3*c*).

These results demonstrate the potential of using oligonucleotides to self-assemble inorganic nanoparticles into aggregates that are discrete, well-defined, homogeneous and soluble. Further improvements in the methodology, particularly shorter and more rigid linkers and soluble, stable ligands for nanocrystals other than Au, are required before the full range of physical phenomena expected in coupled nanocrystal systems can be investigated. However, this approach should allow the chemical synthesis of complex mixtures of nanocrystals of differing sizes and compositions with the size of complete aggregate limited only by the degree of control over the nucleic acid template. □

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- Alivisatos, A. P. *Science* **271**, 933–937 (1996).
- Bawendi, M. G., Steigerwald, M. L. & Brus, L. E. *Annu. Rev. Phys. Chem.* **41**, 477–496 (1990).
- Weller, H. *Angew. Chem. Int. Edn. Engl.* **32**, 41–53 (1993).
- Tolbert, S. H. & Alivisatos, A. P. *Annu. Rev. Phys. Chem.* **46**, 595–625 (1995).
- Wang, F. R. *et al. Phys. Rev. Lett.* **75**, 705–708 (1995).
- Murray, C. B., Norris, D. J. & Bawendi, M. G. *J. Am. Chem. Soc.* **115**, 8706–8715 (1993).
- Littau, K. A., Szajowski, P. J., Muller, A. J., Kortan, A. R. & Brus, L. E. *J. Phys. Chem.* **97**, 1224–1230 (1993).
- Guzelian, A. A. *et al. J. Phys. Chem.* **100**, 7212–7219 (1996).
- Schmid, G. *Chem. Rev.* **92**, 1709–1727 (1992).
- Haneda, K. *Can. J. Phys.* **65**, 1233–1241 (1987).
- Spanhel, L., Weller, H. & Henglein, A. *J. Am. Chem. Soc.* **109**, 6632–6635 (1987).
- Gopidas, K. R., Bohorquez, M. & Kamat, P. V. *J. Phys. Chem.* **94**, 6435–6440 (1990).
- Brust, M., Bethell, D., Schiffrin, D. J. & Kiely, C. J. *Adv. Mater.* **7**, 795–797 (1995).
- Lawless, D., Kapoor, S. & Meisel, D. *J. Phys. Chem.* **99**, 10329–10335 (1995).
- Pag, X. *et al. Angew. Chem.* (submitted).
- Peschel, S. & Schmid, G. *Angew. Chem. Int. Edn. Engl.* **34**, 1442–1443 (1995).
- Whetten, R. L. *et al. Adv. Mater.* **8**, 428–433 (1996).
- Andres, R. P. *et al. Science* **272**, 1323–1325 (1996).
- Klein, D. L., McEuen, P. L., Bowen-Katari, J. E., Roth, R., Alivisatos, A. P. *Appl. Phys. Lett.* **68**, 2574–2576 (1996).

- Covin, V. L., Goldstein, A. N., Alivisatos, A. P. *J. Am. Chem. Soc.* **114**, 5221–5230 (1992).
- Fendler, J. H., Meldrum, F. C. *Adv. Mater.* **7**, 607–632 (1995).
- Peng, X. *et al. J. Phys. Chem.* **96**, 3412–3416 (1992).
- Murray, C. B., Kagan, C. R. & Bawendi, M. G. *Science* **270**, 1335–1338 (1995).
- Vossmeier, T. *et al. Science* **267**, 1476–1479 (1995).
- Herron, N., Calabrese, J. C., Farneth, W. E. & Wang, Y. *Science* **259**, 1426–1428 (1993).
- Bentzon, M. D., van Wonerghem, J., Morup, S., Tholen, A. & Koch, C. J. W. *Phil. Mag. B* **60**, 169–178 (1989).
- Seeman, N. C. *Mater. Res. Soc. Symp. Proc.* **292**, 123–135 (1993).
- Niemeyer, C. M., Sano, T., Smith, C. L. & Cantor, C. R. *Nucleic. Acids Res.* **22**, 5530–5539 (1994).
- Zuckermann, R. N., Corey, D. R. & Schultz, P. G. *Nucleic. Acids Res.* **15**, 5305–5321 (1987).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY, 1989).

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CORRESPONDENCE should be addressed to A.P.A. or P.G.S.

Detection of postseismic fault-zone collapse following the Landers earthquake

Didier Massonnet*, Wayne Thatcher† & Hélène Vadon*

* Centre National d'Etudes Spatiales, 18 Avenue E. Belin, 31055 Toulouse, France

† United States Geological Survey, 345 Middlefield Road, Menlo Park, California 94025, USA

STRESS changes caused by fault movement in an earthquake induce transient aseismic crustal movements in the earthquake source region that continue for months to decades following large events¹⁻⁴. These motions reflect aseismic adjustments of the fault zone and/or bulk deformation of the surroundings in response to applied stresses^{2,5-7}, and supply information regarding the inelastic behaviour of the Earth's crust. These processes are imperfectly understood because it is difficult to infer what occurs at depth using only surface measurements², which are in general poorly sampled. Here we push satellite radar interferometry to near its typical artefact level, to obtain a map of the postseismic deformation field in the three years following the 28 June 1992 Landers, California earthquake. From the map, we deduce two distinct types of deformation: afterslip at depth on the fault that ruptured in the earthquake, and shortening normal to the fault zone. The latter movement may reflect the closure of dilatant cracks and fluid expulsion from a transiently over-pressured fault zone⁶⁻⁸.

We measure the deformation around the 1992 rupture zone over three years by producing interference patterns from the difference of two synthetic-aperture radar (SAR) images acquired by the ERS-1 satellite. This geodetic technique can reveal surface phase changes⁹ and displacements due to earthquakes¹⁰⁻¹⁵, glaciers¹⁶ or volcanoes¹⁷ with centimetre, and sometimes millimetre, accuracy. The interferogram is a contour map of the change in range, or the component of the displacement vector which points toward the satellite.

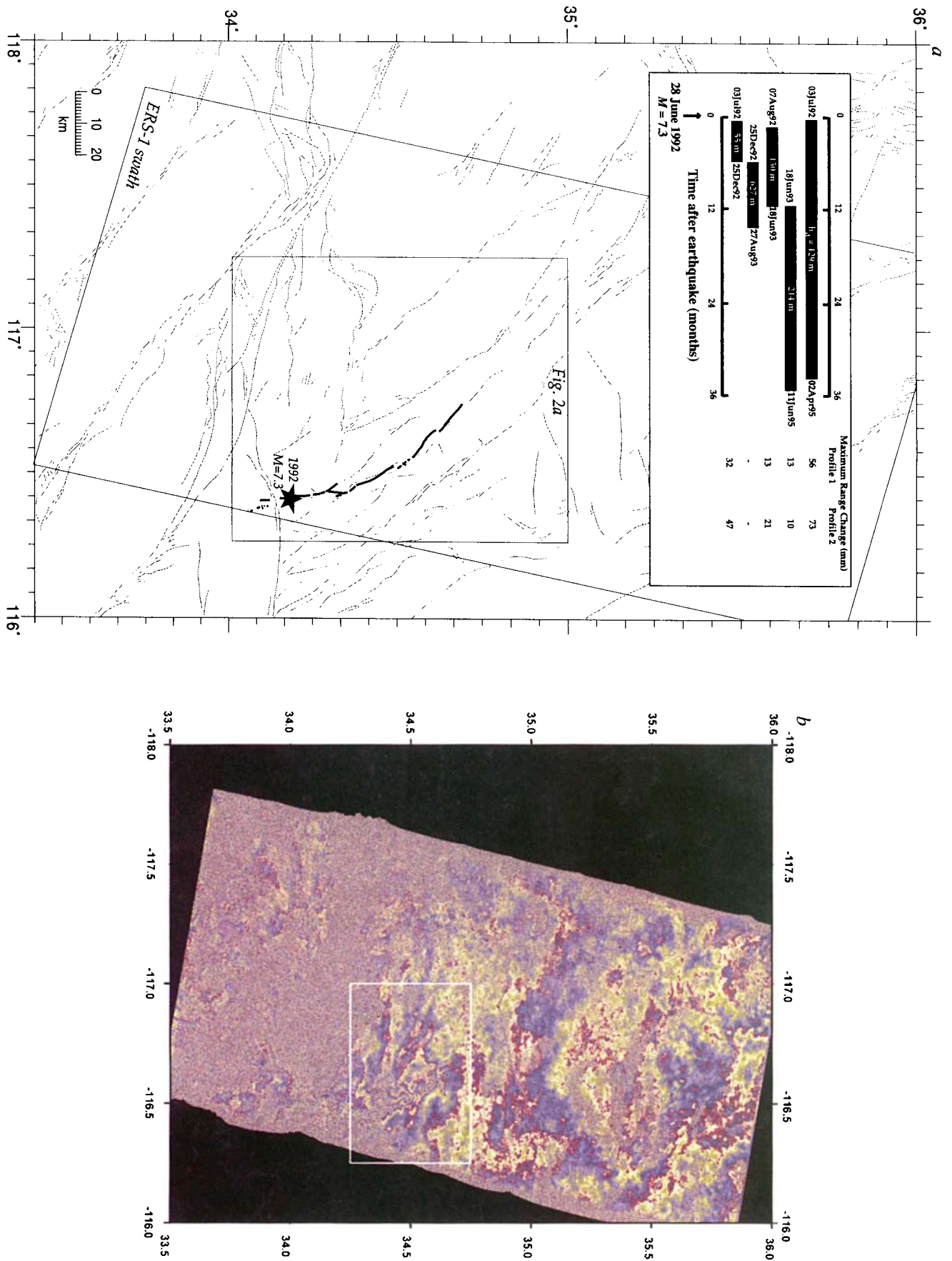
The techniques used to calculate the interferograms have been used in our three previous papers^{10,11,17}. They require a digital elevation model (DEM)¹⁸ to calculate the effect of topography and can obtain a result using any pair of radar images from the same orbital position. The DEM is used in four critical steps of the processing by allowing: (1) prediction of the stereoscopic geometric changes occurring between the radar images, which allows their accurate registration regardless of local decorrelation, (2) selection, depending on the local slope, of filters which optimize fringe quality in the interferogram formation, (3) simulation of the topographic fringe pattern in order to subtract it from the interferogram, (4) display of the results in map coordinates. In our previous papers, orbital effects were eliminated by adjusting the orbital parameters under the geophysical assumption of negligible deformation at the four corners of the interferograms, a condition which might possibly be violated for interseismic deformation of regional scale. In this study, the adjustment was obtained by minimizing phase gradient in selected portions of the interferograms.

The ERS-1 satellite acquired several radar images during its first 35-day orbital cycle (April 1992–December 1993). The use of other orbital cycles in 1994 and the first three months of 1995 prevented us from applying the technique because these cycles did not observe the Landers area (three-day cycle) or were too long to create pairs of images acquired from sufficiently closely spaced tracks (shifted 168-day cycles). ERS-1 came back on its previous 35-day cycle just before the successful launch of its ERS-2 sister

satellite in April 1995. We have selected four images to study on the basis of their altitude of ambiguity¹⁹ and time separation. Use of multiple images thus permits us to rule out potential artefacts, which can have the same amplitude as the useful signal²⁰, itself maximized by the elapsed time. The successful correlation between the first (3 July 1992) and last (2 April 1995) images was anticipated despite the 33-month time lapse because we had already sampled, with good results, a full yearly seasonal cycle with ERS-1 interferometry at this site¹¹. Following our previous convention¹¹ of counting from the date of the Landers main shock (28 June 1992), we call the 'long' interferogram the result of this correlation, which maps range changes from day 5 to day 1008 (1,003 days). The 'medium' interferogram covers the period from day 40 to 355 (315 days). The 'combined' interferogram, which could not be produced by any direct combination of radar images, is the integer sum of the two²¹ (Fig. 1b). Because the two orbits used to create each interferogram are not strictly identical, the single interferograms each contain a contribution from uncorrected topography, resulting from the limited accuracy of the DEM. The long and medium interferograms are almost equally sensitive to topography, but with opposite sign, owing to the complementary offsets of the orbital pairs that make up each interferogram; accordingly the combined interferogram is almost completely insensitive to topography. In contrast, any ground deformation occurring in the time elapsed between the acquisition dates of the images forming the medium interferogram is also present in the long interferogram, whose image acquisition dates frame the former. The contribution of such a signal is therefore doubled in the combined interferogram. A clear example is given by the $M_w = 5.4$ event^{11,22} (Fig. 2b), but this doubling also applies to most of the postseismic signal, contributing to its clarity.

A qualitative analysis is first performed on the interferograms in order to discriminate between various effects²⁰. The interferograms contain the signature of atmospheric heterogeneity, which creates about one cycle of contamination²⁰. Atmospheric artefacts attached to any given radar image cannot appear on both interferograms, as they are made from four independent radar images. On the contrary, we recognize ground deformation on both long and medium interferograms, albeit with a different amplitude, in the area of the Landers fault (Fig. 1b). It reaches up to two closed

FIG. 1 a, Location map. Thin lines denote known faults²⁷; heavy lines, the surface rupture. The two northwest-striking faults to the west of the surface rupture are the Old Woman and Lenwood faults, across which surface afterslip is identified in Fig. 2a (see text). Large, inclined box delimits the 120 × 275 km area covered by the interferograms. The radar antenna, at 785 km altitude, points across this swath at an azimuth of N77° W with an average angle of incidence of 23°. The interferometric sensitivity to various ground displacements is expressed by the unitary vector (east, north, up): $\mathbf{E} = 0.333$; $\mathbf{N} = -0.07$; $\mathbf{U} = 0.94$. The smaller box is the location of Fig. 2a. The star denotes the epicentre of the 1992 earthquake. Inset, bar chart showing the interferograms used in this study as a function of time. The number on each bar is h_a , the altitude of ambiguity, equal to the amount of error in the DEM which produces one artefact fringe. The elevation model was produced by the US Defense Mapping agency which calls it a Digital Terrain Elevation Data, Level 1 (DTED-1), and is distributed by the US Geological Survey²⁸. b, 'Combined' interferogram constructed by adding two interferograms made of radar images acquired: (1) on 3 July 1992 (orbit 5053) and 2 April 1995 (orbit 19425). The altitude of ambiguity of the pair is 129 m, implying that a 30-m error in the elevation model would create an error of 7 mm in the interferogram. The successful correlation of two images separated by almost three years was expected on this site¹¹. (2) On 7 August 1992 (orbit 5554) and 18 June 1993 (orbit 10063), which maps deformation in the period 40 to 355 days after the 28 June 1992 earthquake. The altitude of ambiguity of this interferogram is -128 m. No pair of radar images could create this interferogram. The effective altitude of ambiguity is more than 16,000 m, suppressing all topographic residuals. An uncorrected topography of 129 times 128 m, or 16,512 m, would create 128 fringes in the first interferogram and -129 in the second. The largest topographic structures on Earth, even uncorrected, would not create a single topographic fringe in the sum of these interferograms. The white box outlines the part of the interferogram shown in Fig. 2a.



fringes. Figure 2a shows an enlarged portion of the combined interferogram of Fig. 1b near the 1992 Landers earthquake rupture, and Fig. 2b is a further enlarged contour map of the interference fringes (range change) near the 1992 fault.

Two distinct patterns of deformation are highlighted in Fig. 2b. Pattern 1, on the northern and central parts of the surface rupture, consists of a region of range increase southwest of the fault and range decrease to the northeast. It resembles the coseismic fringe

pattern^{10,23}, but at much reduced amplitude and without any discontinuity across the fault, immediately suggesting continued fault slip that does not reach the earth's surface. Pattern 2, on the southern half of the 1992 fault, shows a much more localized region of range decrease lying within a few kilometres of the Landers fault zone. Local truncations or high gradients in interference fringes near faults indicate near-surface slip at these locations, but these features are exceptional rather than typical.

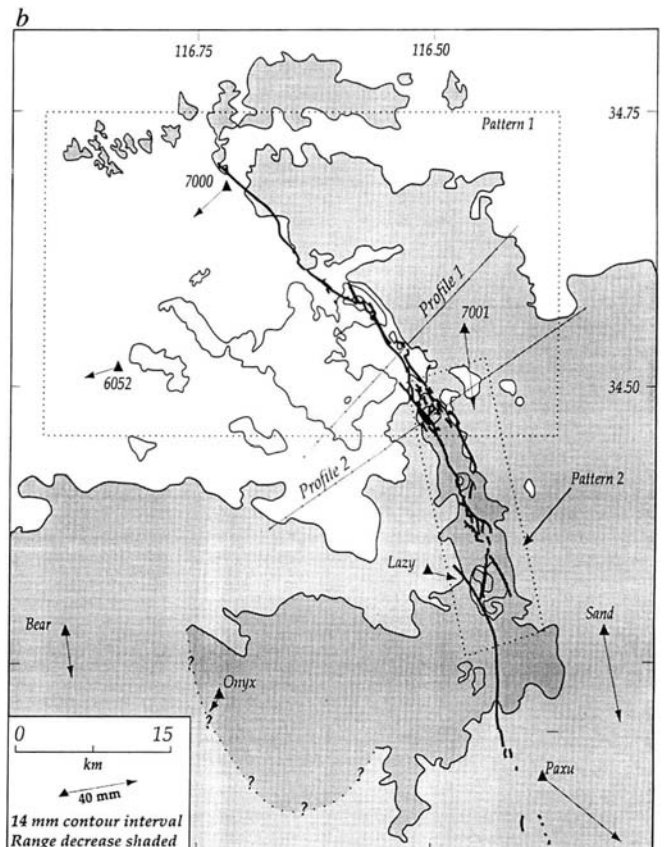
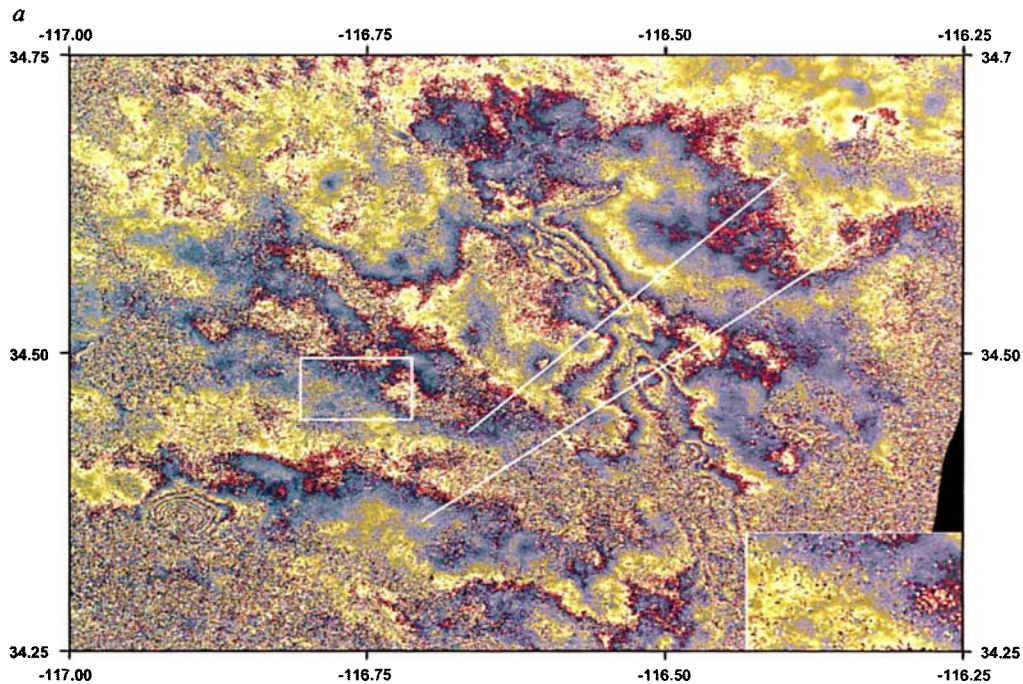


FIG. 2 a, Detail of the combined interferogram. Heavy straight lines show the two profiles used for the quantitative part of this study. One cycle of colour (red-yellow-blue) corresponds to one interferometric fringe, equivalent to 28 mm of change in the range between the satellite and the reflector on the ground (along the line of sight described above by the **E, N, U** vector). Inset, denoted by the white box, is an enlarged view showing the offset across the Old Woman fault, indicated by the colour change from yellow to blue on a nearly linear trend. b, Detail of contours for the interferogram of a. Distance is scaled on a cartographic reference frame in order to show accurately fault and displacement vector orientations. Thick lines denote Landers fault. Locations of two deformation patterns discussed in the text are outlined by dotted rectangles, and locations of profiles plotted in Fig. 3 are also shown. Filled triangles locate GPS stations and arrows show inferred displacements during the six months following the Landers earthquake²⁶. Converting these GPS displacements to equivalent range changes (see text), we obtain the following values (in mm), where a negative sign implies range decrease: station 7000 (10), station 7001 (-2), station 6052 (10), Lazy (-7), Bear (0), Onyx (2), Sand (2), Paxu (-23).

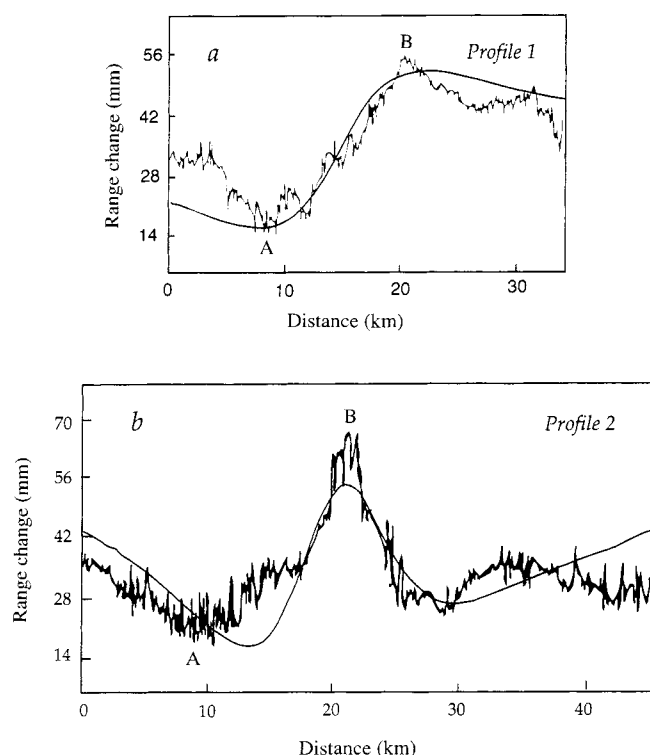


FIG. 3 *a*, Profile 1; *b*, profile 2. The irregular line shows data; the smooth curves are model fits (see text). The maximum amplitude was obtained between points A and B in the profiles.

We also observe slip across the Lenwood and Old Woman faults (Fig. 1*a*) as discontinuities^{11,23} in the fringe pattern in Fig. 2*a*, and because no earthquake occurred there in the time spanned by the interferogram, these motions must have been aseismic.

Adjacent range-change profiles (locations shown in Fig. 2*b*) that pass through each of these deformation patterns are shown in Fig. 3 along with model profiles that have been fitted to them. Profile 1 is matched well by a model (model 1) with 90 cm of right-lateral slip on a 5-km-wide fault strip lying between 6 and 11 km on the same plane that ruptured in the 1992 earthquake. The slight mismatch between model and data near the southwestern edge of the profile is due to an offset across the Lenwood fault. It is caused by the small amount of postseismic surface slip on this fault like that documented by our previous work¹¹. The profile lies in the region where coseismic displacement was a maximum^{24,25}, suggesting continued slip on the same fault patch. Movements must be dominantly aseismic because slippage is much more than can be accounted for by aftershocks of the 1992 event. Postseismic slip at depth has been observed for many earthquakes but it is commonly confined to the down-dip edge of the coseismic rupture plane rather than the rupture plane itself³.

Profile 2 is fitted acceptably by a conceptually very different model. Following recent models of fault-zone crack growth and fluid flow^{6,7}, and similar postseismic deformation modelling⁸, we assume that earthquake motions open dilatant cracks in a fluid-saturated fault zone, resulting in time-dependent post-earthquake crack closure and fluid flow. Such crack closure and fluid expulsion may be crudely modelled by elastic dislocation models in which the 'slip' is normal to the fault surface, approximating postseismic collapse (volume decrease) within the fault zone. In Fig. 3*b* we apply such a model, with 30 cm of fault closure on the same fault strip used in model 1 (5 km wide lying between 6 and 11 km depth). But as profile 2 also includes movements due to model 1 (see profile 2 location in Fig. 2*b*), we must superpose a fraction of the model 1 motion onto profile 2; in Fig. 3*b* we have added 70% of the model 1 deformation to that of model 2. The procedure, done

by trial-and-error, is clearly subjective and non-unique, indicating some uncertainty in the derived parameters of model 2, particularly the depth and slip magnitude of the equivalent dislocation.

Profiles such as those in Fig. 3 can easily be obtained for all of the postseismic interferograms computed in this area (see Supplementary Information). Although the postseismic pattern is of lower amplitude and is not always recognized easily, the general pattern of signal persists from one interferogram to another whereas the background noise is, by contrast, incoherent. (See Supplementary Information for a random profile.) We plotted the amplitude of range change between the points where it is maximum (points A and B on Fig. 3*a* and *b*). If we exclude the interferograms using the acquisition of 28 August 1993, where a large propagation path contribution has already been described²⁰, we observe that the data fit a logarithmic law reasonably well, with a general shape given by $X \log(t) + Y$, where t is the time elapsed since the Landers event in days. Because interferograms give only a difference between two, or more, radar acquisition dates, Y cannot be determined. The amplitude factor X is found to be 22.5 mm for profile 1 of Fig. 3*a*, and 30 mm for profile 2 of Fig. 3*b*. The r.m.s. uncertainty of X is 5 mm for both profiles.

Our models are consistent with the Global Positioning System (GPS) data gathered at 19 ground stations in the six months following the Landers earthquake²⁶. However, only a limited subset of these data are relevant to our models. Horizontal displacement vectors derived from the GPS data and corrected for inferred interseismic movements²⁶ are plotted for the eight stations located within the frame of Fig. 2*b*. The five stations that lie within 20 km of the Landers rupture show general features of the postseismic deformation, with roughly fault-parallel movements except near the ends of the rupture. Because of end effects, near-fault complexities, and likely along-strike variations in postseismic slip, station displacements both close to the rupture (Lazy) and near its termination points (station 7000, Sand, Paxu) are of limited utility in comparing with our model results. Station 7001, which lies close to our profiles and about 8 km from the fault, provides the most definitive check. We first use the maximum amplitudes observed on the 3 July to 25 December 1992 interferogram profiles (Fig. 1*a* inset) to infer the contributions of models 1 and 2 in the six months following the earthquake. Assuming equal contributions from each model we compute a horizontal displacement at station 7001 of 49 mm on an azimuth of 162°, which compares with the GPS values of 36 mm along 171°, acceptable agreement considering the assumptions used in both calculations. Model 1 has three times the slip of model 2, so the effect of model 2 is to rotate the displacement vector slightly (20°) towards the fault, as is observed. The fault-normal displacement of station Lazy is consistent with fault-zone collapse (model 2), but the site does lie within a few kilometres of a secondary splay of the surface rupture. In addition, any model 1 slip required to explain fault-parallel movements of station Sand would introduce a more northerly component into the displacement field at station Lazy.

We can also perform a limited check between the GPS data and our interferometric observations. GPS measurements of vertical displacement are too inaccurate to use here, but if we assume no significant vertical motions occurred, we may convert the GPS station displacements into range-change values. Scaling these values to account for the differing time intervals between GPS and radar data shown in Fig. 2*b*, we obtain the inferred range changes listed in the figure legend. The gradients in range change obtained in this way agree acceptably with those obtained interferometrically. For example, the average difference between points on the unshaded part of the Fig. 2*b* contour map (stations 7000, 6052) and those on it (station 7001, Lazy, Bear, Onyx, Sand, Paxu) is 14 mm, about equal to the one contour interval difference in range change shown on the interferogram.

We found that synthetic interferograms computed using the postseismic-slip model derived²⁶ from inversion of the GPS data are quite inconsistent with our SAR images. This disagreement is not surprising given the admitted non-uniqueness of the model²⁶

and the very incomplete spatial sampling of the deformation field by the GPS measurements. The comparison illustrates the difficulty of unravelling the mechanisms responsible for observed deformation with necessarily limited conventional survey measurements, especially when, as here, more than one process is operating. Our result not only shows the unique capability of SAR interferometry to provide the necessary complete mapping of the postseismic deformation field but, as importantly, it casts new light on poorly understood lithospheric relaxation processes. Given the demonstrated sensitivity of the method, we can expect that new SAR measurements of postseismic deformation will play an important role in more fully elucidating these processes. The result also provides grounds for optimism that the even fainter deformation signal associated with inter-earthquake elastic strain accumulation can also be detected using radar interferometry. $\square$

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1. Okada, A. & Nagata, T. *Bull. Earthq. Res. Inst. Tokyo Univ.* **31**, 151–167 (1953).
2. Thatcher, W. J. *geophys. Res.* **88**, 5893–5902 (1983).
3. Thatcher, W. R. *Soc. N.Z. Bull.* **24**, 245–272 (1986).
4. Savage, J. C. & Plafker, G. J. *geophys. Res.* **96**, 4325–4335 (1991).
5. Thatcher, W. & Rundle, J. B. J. *geophys. Res.* **89**, 7631–7640 (1984).
6. Sleep, N. H. & Blanpied, M. L. *Nature* **359**, 687–692 (1992).
7. Byerlee, J. *Geology* **21**, 303–306 (1993).
8. Savage, J. C., Lisowski, M. & Svarc, J. L. J. *geophys. Res.* **99**, 13757–13765 (1994).

9. Gabriel, A. K., Goldstein, R. M. & Zebker, H. A. J. *geophys. Res.* **94**, 9183–9191 (1989).
10. Massonnet, D. et al. *Nature* **364**, 138–142 (1993).
11. Massonnet, D., Feigl, K. L., Rossi, M. & Adragna, F. *Nature* **369**, 227–230 (1994).
12. Peltzer, G. & Rosen, P. *Science* **268**, 1333–1336 (1995).
13. Massonnet, D. & Feigl, K. L. *Geophys. Res. Lett.* **22**, 1541–1544 (1995).
14. Murakami, M., Tobita, M., Saito, T. & Masharu, H. J. *geophys. Res.* **101**, 9183–9191 (1996).
15. Massonnet, D., Feigl, K. L., Vadon, H. & Rossi, M. *Geophys. Res. Lett.* **23**, 969–972 (1996).
16. Goldstein, R. M., Engelhardt, H., Kamb, B. & Frolich, R. M. *Science* **262**, 1525–1530 (1993).
17. Massonnet, D., Briole, P. & Arnaud, A. *Nature* **375**, 567–570 (1995).
18. Massonnet, D. *Etude de Principe d'une Détection de Mouvements Tectoniques par Radar* (Int. Memo No. 326, Centre National d'Etudes Spatiales, Toulouse, 1985).
19. Massonnet, D. & Rabaute, T. *IEEE Trans. Geosci. Rem. Sensing* **31**, 455–464 (1993).
20. Massonnet, D. & Feigl, K. L. *Geophys. Res. Lett.* **22**, 1537–1540 (1995).
21. Massonnet, D., Vadon, H. & Rossi, M. H. *IEEE Trans. Geosci. Rem. Sensing* **34**, 489–497 (1996).
22. Feigl, K. L., Sergeant, A. & Jacq, D. *Geophys. Res. Lett.* **22**, 1037–1040 (1995).
23. Zebker, H. A., Rosen, P., Goldstein, R. M., Gabriel, A. & Werner, C. L. J. *geophys. Res.* **99**, 617–634 (1994).
24. Wald, D. J. & Heaton, T. H. *Bull. seism. Soc. Am.* **84**, 668–691 (1994).
25. Freymüller, J., King, N. E. & Segall, P. *Bull. seism. Soc. Am.* **84**, 646–659 (1994).
26. Shen, Z., Jackson, D., Feng, Y., Kim, M. & Cline, M. *Bull. seism. Soc. Am.* **84**, 780–791 (1994).
27. California Division Mines and Geology *Preliminary Fault Activity Map of California* (Calif. Dept. of Conservation, Sacramento, 1992).
28. US Geological Survey *Digital Elevation Models: Data Users Guide 5 1–34* (US Govt Print. Off., Washington DC, 1993).

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Fingerprint of ozone depletion in the spatial and temporal pattern of recent lower-stratospheric cooling

V. Ramaswamy*, M. D. Schwarzkopf* & W. J. Randel†

* NOAA/Geophysical Fluid Dynamics Laboratory, Princeton, New Jersey 08542, USA

† National Centre for Atmospheric Research, PO Box 3000, Boulder, Colorado 80307, USA

OBSERVATIONS of air temperatures in the lower stratosphere from 1979 to 1990 reveal a cooling trend that varies both spatially and seasonally¹. The possible causes of this cooling include changes in concentrations of ozone or of other greenhouse gases^{2,3}, and entirely natural variability, but the relative contributions of such causes are poorly constrained. Here we incorporate the observed decreases in stratospheric ozone concentrations⁴ over the same period into a general circulation model of the atmosphere, to investigate the role of the ozone losses in affecting patterns of temperature change. We find that the simulated latitudinal pattern of lower-stratospheric cooling for a given month through the decade corresponds well with the pattern of the observed decadal temperature changes. This result confirms the expectation, from simpler model studies^{2,5}, that the observed ozone depletion exerts a spatially and seasonally varying fingerprint in the decadal cooling of the lower stratosphere, with the influence of increases in concentrations of other greenhouse gases being relatively small. As anthropogenic halocarbon chemicals are important causes of stratospheric ozone depletion^{2,3}, our study suggests a human influence on the patterns of temperature change in the lower stratosphere over this 11-year period.

The ozone molecule, through its absorption of ultraviolet and visible solar radiation and absorption and emission of longwave radiation, is an important component in the stratospheric energy balance. Thus, a loss of ozone is expected to perturb the climate of the stratosphere^{6–12}. The version of the GFDL 'SKYHI' general circulation model (GCM) used here to assess such perturbations, has a latitude–longitude resolution of $\sim 3^\circ \times 3.6^\circ$ and has 40

layers extending from the surface to 80 km (ref. 13). The model has fixed cloud distributions in the troposphere and climatologically varying sea surface temperatures. The total air-column values of the zonal, monthly-mean ozone losses over the past decade are taken from satellite measurements⁴. The observed ozone losses occur principally in the lower stratosphere (up to ~ 23 km, depending on latitude) of the middle (20° – 60°) to high (60° – 90°) latitude zones almost throughout the year². There exists some uncertainty concerning the exact vertical profile of the loss, particularly around the tropopause region, which is important for the radiative perturbations^{2,3,14,15}. Following other satellite¹⁶ and ground-based observations², we assume a simple vertical profile of the loss, with the depletion extending from the model's climatological 'tropopause' level to ~ 7 km above it and with a constant percentage loss within this altitude range¹². The model's climatological 'tropopause' pressure level is specified to vary linearly with latitude, from 92 hPa (~ 17 km) at the Equator, to 180 hPa (~ 12 km) at 45° and 280 hPa (~ 9 km) at the poles. This definition, together with the assumed vertical loss profile, is aimed at describing the influence of ozone losses that occur at altitudes just above the troposphere–stratosphere boundary. The GCM's 'control' and perturbation experiments are each run for 10 years, and the time-averaged response over this period is analysed.

The GCM's zonally and annually averaged profile of the temperature response due to the radiative perturbation (Fig. 1) shows a cooling in almost the entire lower-stratospheric region. The cooling is greater in the northern than in southern middle latitudes owing to larger ozone losses (hence, a greater radiative perturbation) occurring more towards the Equator in the Northern Hemisphere⁴. There is a cooling even in those regions where there are no ozone losses imposed in the model, for example the lower stratosphere between the Equator and 15° latitude. Above the domains with both ozone loss and lower-stratospheric cooling, there is a warming, particularly evident at the Southern Hemisphere high latitudes. These features are a manifestation of the dynamical changes in which the ozone-induced radiative perturbations alter the circulation in the global lower stratosphere, increasing the net adiabatic cooling in the tropics and the subsidence-related heating at higher latitudes. The dynamical changes are qualitatively similar to those found in a GCM study of the Antarctic ozone hole¹⁰. There is also a cooling below the ozone-loss region in the global upper troposphere, in part due to reduced infrared emission from the stratosphere. Figure 1 indicates that the annual-mean response is highly significant (that is, it has a high signal-to-noise ratio) between ~ 13 and 21 km in the $\sim 20^\circ$ – 50°

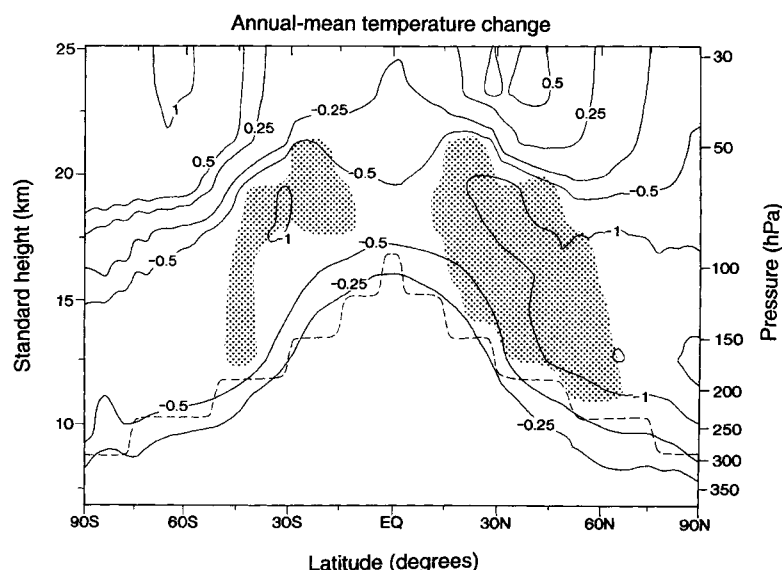


FIG. 1 Change in the zonally and annually averaged vertical profile of temperature, as simulated by a general circulation model (GCM) due to the ~1979–90 observed global ozone losses^{4,16}. Shaded areas in the figure show statistical significance at the 99% confidence level, as determined using a Student's *t*-test. The assumed 'tropopause' level (thin, dashed line) in the model is also indicated.

latitude belt. The changes at high latitudes ($>60^\circ$) fail the significance test because of large interannual variability in those regions, a feature corroborated by observations^{17,18}.

The latitude-month pattern of the decadal (~1979–90) temperature change simulated by the model, in the altitude range of the ozone changes (Fig. 2a), is compared with that derived from satellite observations¹ (Fig. 2b) of the lower stratosphere. The satellite-derived general cooling trend in the lower stratosphere is well supported by the radiosonde observations^{19–21}. The observed decadal cooling in the middle-to-high latitudes exceeds 0.5 K for most of the year, reaching 2.5 K in the polar spring in both hemispheres; these values constitute substantial changes over a relatively short period. The simulated result also exhibits a marked cooling over a broad latitude–month domain, especially in the middle and high latitudes. The middle latitudes in Fig. 2a and b show a cooling from January to October in the Northern Hemisphere and from September to July in the Southern Hemisphere. The cooling in the middle latitudes of the Northern Hemisphere from about December to July, and in the Southern Hemisphere from about December to May, is statistically significant in both the model and in observations. Near the poles, both the simulations and the observations exhibit a cooling pattern almost throughout the year, with the occurrence of relatively large magnitudes during winter and spring. The simulated cooling in the Antarctic is highly significant during the austral spring (period of the 'ozone hole'), consistent with observations. The cooling in the Arctic during spring does not show a high significance in either Fig. 2a or b owing to a large dynamical variability^{13,18,20}. The simulated cooling in the tropics is not significant for most of the year owing to the small temperature changes. There are some differences in patterns between the simulated and observed trends, especially during certain seasons in the polar regions. In particular, a larger domain of significance is seen in the simulation. This is due to a smaller variability in the model compared with the observations. Uncertainties arise in the simulation owing to incomplete observational knowledge of the vertical profile of global ozone loss near the tropopause, including that in the tropical areas^{2,3,16}. While more comprehensive altitudinal measurements of ozone loss would lead to more precise simulations of temperature change, with cooling perhaps extending to even higher altitudes (for example springtime southern polar latitudes¹⁰), the lower stratosphere region, taken as a whole, can be expected to cool significantly given the magnitude of the observed^{4,16} ozone losses. We conclude that the reasonable consistency of the simulated cooling pattern and magnitudes with those observed, including the regimes of statistically significant changes, coupled with the high correlations noted between

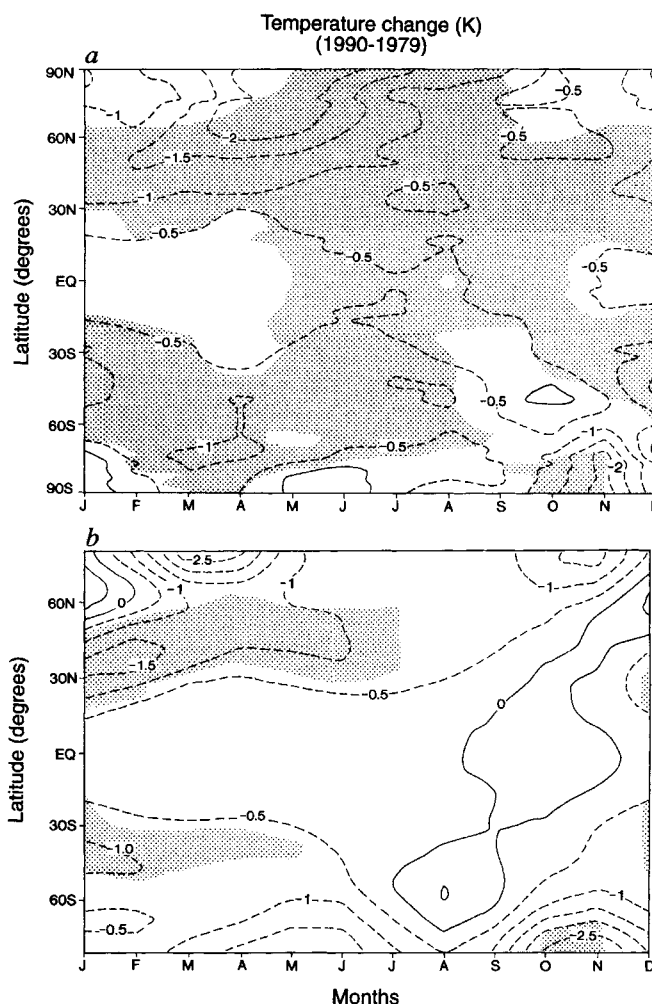


FIG. 2 Zonal, monthly-mean pattern of the lower-stratospheric temperature change over the past decade (~1979–90). a, As simulated by the GCM (90°S to 90°N) due to the observed global ozone depletion^{4,16}; b, as inferred from satellite observations (82.5°S to 82.5°N)¹. The satellite temperature trends are derived for an atmospheric layer essentially comprising the lower stratosphere². The simulated results denote the mean temperature trends in the altitude region of the model where ozone concentration changes occur. Shaded areas denote the statistical significance at the 95% confidence level¹.

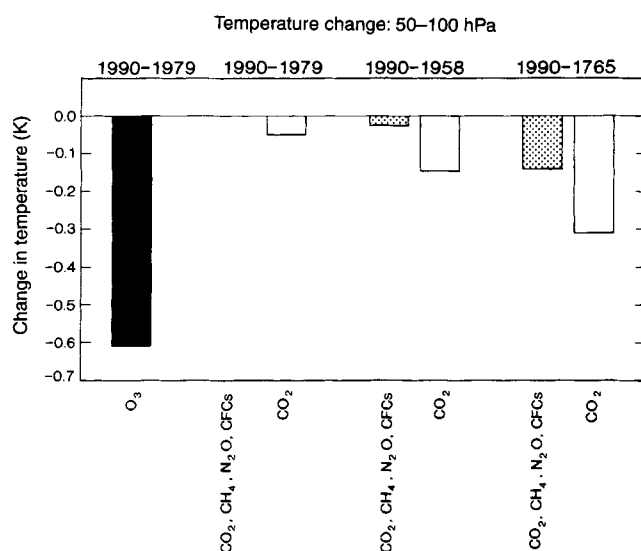


FIG. 3 Computed global, annual-mean temperature changes in the ~50–100 hPa (~16–21 km) lower-stratospheric region due to different trace-gas changes. The result for ozone corresponds to the ~1979–90 losses, and is computed using a GCM. The results for CO₂ alone, and for all the well mixed greenhouse gases (CO₂, CH₄, N₂O and CFCs) are calculated using a one-dimensional, radiative-convective model for three different periods: 1979 to 1990, 1958 to 1990 and 1765 to 1990. Note that the result for all the well mixed gases in the period 1979–90 is too small to be distinguishable from zero.

observed temperature changes and ozone losses¹, confirms the notion that ozone depletion has caused a substantial spatially and seasonally dependent effect in the lower stratosphere over the past decade.

To evaluate the importance of ozone depletion relative to changes in the concentrations of other greenhouse gases that are well mixed (CO₂, CH₄, N₂O, chlorofluorocarbons), we employ a radiative-convective model and determine the global, annual-mean temperature change in the lower stratosphere due to the known increases in their concentrations. The model's radiation and convection schemes follow those used in earlier studies^{12,22}. The calculations are performed for three different periods²³: ~1979 to 1990, 1958 (ref. 24) to 1990 (the period since routine CO₂ measurements began) and 1765 to 1990 (the period since the beginning of the industrial era). In Fig. 3 the global, annual-mean GCM temperature change due to the decadal ozone losses in the ~50–100 hPa (~16–21 km) lower-stratospheric region is compared with the corresponding effects due to increases in CO₂ only, and all well mixed greenhouse gases taken together. The global-mean decadal cooling due to ozone is ~0.6 K, with the middle latitudes (Fig. 1) making a substantial contribution; the

value is comparable to the reported decadal trends^{3,25}. Although the increase in CO₂ alone since 1765 yields a cooling of ~0.3 K, inclusion of the other well mixed gases, which together tend to warm the tropopause region²⁶ gives a cooling of ~0.15 K. The overall cooling effect in the lower stratosphere due to increases in the well mixed greenhouse gases contrasts with their warming effect on the surface^{24,26}. It is thus clear that the computed 1979–90 ozone effect on lower-stratospheric temperature outweighs the effects of changes in other gases, not only over the past 10–30 years, but also over the past two centuries. This sharp contrast in the effects of ozone in relation to the other gases is qualitatively similar to other global-mean estimates^{3,11,27}.

Possible secular changes in other radiatively active species²², including stratospheric volcanic aerosols and water vapour²⁸, are estimated to contribute considerably smaller decadal effects than the stratospheric ozone loss. Information on decadal changes in clouds is insufficient to estimate their influence. There is little evidence to suggest that forcings from the troposphere (for example, sea surface temperature changes²⁹) or natural climate variability (gauged from the 'control' simulation here and in other analyses^{30,31}) have significantly influenced the decadal global lower-stratospheric temperature change, although in the absence of rigorous long-term observations, a precise estimate of their contributions cannot be obtained. The knowledge available at present, and current model simulations strongly suggest that the 1979–90 ozone loss has played a dominant role in the latitude-month pattern of cooling observed in the global lower stratosphere. It is noted that ozone loss has also been reported²⁷ for the 1970s, although the observations then did not span the globe. The 1970s losses are also estimated^{11,27} to have contributed significantly to the observed cooling^{19–21} during that decade.

An ozone-induced cooling of the lower stratosphere implies a reduction in the longwave radiative emission from the stratosphere into the troposphere. This is a mechanism that leads to a negative radiative forcing of the surface-troposphere system^{2,3,12}. The dynamically induced cooling of the tropical lower stratosphere, which we have found, suggests a negative surface-troposphere forcing at the low latitudes. This is a feature that could not be predicted by earlier calculations, and which would enhance the global-mean negative forcing caused by the observed stratospheric ozone loss^{2,12}.

Because of the connection with the emissions of man-made halocarbon chemicals^{2,3}, the depletion of ozone and its spatial and temporal fingerprint on global lower-stratospheric temperatures becomes a major anthropogenic component of stratospheric climate change—one that has occurred on a much shorter time-scale (~one decade) than the effects due to the well mixed greenhouse gases (several decades). Further, the ozone-loss-induced spatial and seasonal cooling near the stratosphere-troposphere boundary becomes an important factor to be considered in the search for anthropogenic effects on the vertical temperature profile record^{11,30}. □

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- Randel, W. J. & Cobb, J. B. *J. Geophys. Res.* **99**, 5433–5447 (1994).
- Scientific Assessment of Ozone Depletion: 1991, *Global Ozone Research and Monitoring Project Rep. No. 25* Ch. 7 & 8 (WMO, Geneva, Switzerland, 1992).
- Scientific Assessment of Stratospheric Ozone Change: 1994, *Global Ozone Research and Monitoring Project Rep. No. 37* Ch. 8 (WMO, Geneva, Switzerland, 1995).
- Stolarski, R. S., Bloomfield, P., McPeters, R. D. & Herman, J. R. *Geophys. Res. Lett.* **18**, 1015–1018 (1991).
- McCormack, P. & Hood, L. L. *Geophys. Res. Lett.* **21**, 1615–1618 (1994).
- Ramanathan, V. & Dickinson, R. E. *J. Atmos. Sci.* **36**, 1084–1104 (1979).
- Feis, S. B., Mahiman, J. D., Schwarzkopf, M. D. & Sinclair, R. W. *J. Atmos. Sci.* **37**, 2265–2297 (1980).
- Shine, K. P. *Geophys. Res. Lett.* **13**, 1331–1334 (1986).
- Kiehl, J. T., Boville, B. A. & Briegleb, B. P. *Nature* **332**, 501–504 (1988).
- Mahiman, J. D., Pinto, J. P. & Umscheid, L. J. *J. Atmos. Sci.* **51**, 489–508 (1994).
- Hansen, J. et al. *Clim. Change* **30**, 103–117 (1995).
- Ramaswamy, V., Schwarzkopf, M. D. & Shine, K. P. *Nature* **355**, 810–812 (1992).
- Hamilton, K. et al. *J. Atmos. Sci.* **52**, 5–43 (1995).
- Lacis, A., Wuebbles, D. & Logan, J. A. *J. Geophys. Res.* **95**, 9971–9981 (1990).
- Schwarzkopf, M. D. & Ramaswamy, V. *Geophys. Res. Lett.* **20**, 205–208 (1993).
- McCormack, M. P., Velga, R. E. & Chu, W. P. *Geophys. Res. Lett.* **18**, 1015–1018 (1992).
- Randel, W. J. *Techn. Note NCAR/TN-295 + STR* (National Center for Atmospheric Res., Boulder, Colorado, USA, 1987).

- Labitzke, K. & van Loon, H. *J. Meteorol. Soc. Jpn* **73**, 883–889 (1995).
- Oort, A. H. & Liu, H. *J. Clim.* **6**, 292–307 (1993).
- Labitzke, K. & van Loon, H. *J. Meteorol. Soc. Jpn* **72**, 1–10 (1994).
- Angell, J. K. *J. Clim.* **1**, 1296–1313 (1988).
- Ramaswamy, V. & Bowen, M. M. *J. Geophys. Res.* **99**, 18909–18921 (1994).
- Shine, K. P., Derwent, R. G., Wuebbles, D. J. & Morcrett, J.-J. in *Climate Change: The IPCC Scientific Assessment* (eds Houghton, J. T. et al.) 41–68 (Cambridge Univ. Press, 1990).
- Scientific Assessment of Stratospheric Ozone Change: 1985, *Global Ozone Research and Monitoring project Rep. No. 16* Ch. 15 (WMO, Geneva, Switzerland 1986).
- Christy, J. R. *Clim. Change* **31**, 455–474 (1995).
- Ramanathan, V., Cicerone, R. J., Singh, H. B. & Kiehl, J. T. *J. Geophys. Res.* **90**, 5547–5566 (1985).
- Miller, A. J. et al. *Geophys. Res. Lett.* **19**, 929–932 (1992).
- Chanin, M.-L. in *The Role of the Stratosphere in Global Change* (ed. Chanin, M.-L.) 301–317 (NATO ASI Ser. I, Global Environ. Change, Vol. 8, Springer, Berlin, 1993).
- Sun, D.-Z. & Oort, A. H. *J. Clim.* **8**, 1974–1987 (1995).
- Santer, B. et al. *Nature* **382**, 39–46 (1996).
- Vinnikov, K., Robock, A., Stouffer, R. J. & Manabe, S. *Geophys. Res. Lett.* **23**, 1801–1804 (1996).

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CORRESPONDENCE should be addressed to V.R.

Complex burrows of the mud shrimp *Callianassa truncata* and their geochemical impact in the sea bed

W. Ziebis, S. Forster, M. Huettel & B. B. Jørgensen

Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

THE biogeochemical processes and associated microbial communities in the sea floor are stratified¹ and dependent on vertical transport mechanisms. The penetration of oxygen is generally only a few millimetres in coastal sediments², regulated by the dynamic balance between diffusion from the overlying water and rapid consumption within the sea bed. Macrofauna organisms living within the sea bed affect the physical structure of the sea floor, its chemical zonations and the exchange processes across the sediment–water interface^{3,4}. Thalassinidean mud-shrimps are often abundant in tropical and temperate coastal regions^{5–7} and build burrows with a species-specific architecture. The deepest reported burrows reach down to 2.5 m sediment depth⁸. It is difficult to study the activities of these secretive animals and their effect on sediment biogeochemistry without disturbing the sediment system⁹. Here we report the use of a diver observatory within the seabed, along with *in situ* measurements, to assess the geochemical impact of the mud-shrimp *Callianassa truncata* Giard and Bonnier (Decapoda, Thalassinidea), a species that commonly inhabits sandy sediments in the Mediterranean sea.

An interesting underwater landscape created by the mud shrimp, *Callianassa truncata*, was found in a shallow bay off the

coast of the Italian island, Giglio, in the Mediterranean Sea (Fig. 1). Conspicuous mounds and funnels covered the sea bed at an average density of 120 mounds per square metre (± 43 , $n = 240$). One mound and two funnels constitute the surface structure of one burrow system, built and maintained by a single shrimp (Fig. 1).

Polyester resin casts revealed that the intricate burrows have a uniform architecture of horizontal galleries interconnected by vertical shafts (Fig. 2a). The first horizontal gallery is connected to the overlying water¹⁰ and consists of three connected chambers aligned in roughly 10 cm sediment depth (Fig. 2b). From two funnels, slanted inhalant shafts lead to the first spherical chamber. The third chamber is elongated and narrows into a thin exhalant tube, which bends at a right angle straight up to the sediment surface and ends at the top of a conical mound. From the central chamber, the burrow continues vertically downward with 7–10 galleries, each consisting of 2–5 spherical chambers at regular depth intervals and it ends blindly in a single chamber.

At the observed abundances (120 per m²) the extensive burrows, with a mean volume of 60 cm³ (± 5 cm³, $n = 14$), increase the total area of the sediment–water interface by roughly 400%. In the course of burrowing, fine-grained sediment is selectively excavated and ejected to the sediment surface through the thin shaft in the mound. Before ejection, the sand grains are gleaned for organic matter. Coarse grains, possibly too heavy to be ejected, are transported downward and are used for the construction of the chamber walls at deeper burrow levels (Fig. 2a, insert). The selective ejection of fine-grained sand into the overflowing sea water increases the bulk sediment permeability of the inhabited area. As the depth intervals of galleries are very regular for all burrows, the accumulation of coarse sand grains in chamber walls resulted in distinct layers of highly permeable coarse sand. Such high permeability facilitates pore-water flow and is thus an important factor for solute exchange in sediments.

The biogenic surface topography alters the small-scale flow regime of the bottom current¹¹. The boundary flow accelerates

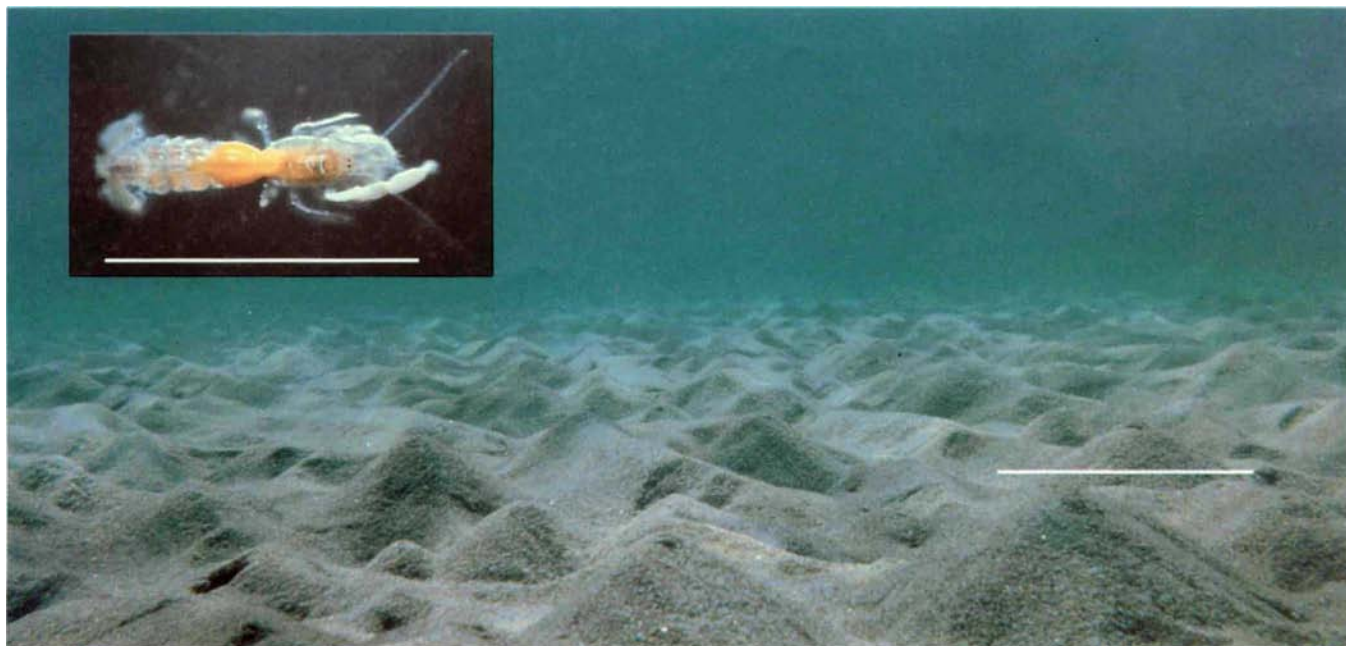


FIG. 1 Sediment surface topography at 5-m water depth created by the burrowing shrimp, *Callianassa truncata* (insert), in the bay of Campese, Isola del Giglio, Italy (42° 20' N, 10° 52' E). Conical mounds with an average height of 4 cm (maximum 9 cm) and funnel-shaped depressions (average depth 4 cm, maximum 6 cm) cover the sea bed between 2 and 15 m water depth in the entire bay. The upper 10 cm of bulk sediment consists of organically poor sand (0.2% dry weight) which is well sorted with a median grain size of 350 μ m. The volcano-shaped mounds consist of finer

sand (median grain size, 200 μ m) than the ambient sediment and are created from discarded sediment which is ejected by the animals at the excurrent openings of their burrows. On average, 2–3 kg of sand per m² and per day were ejected up to 6 cm up into the bottom-water current, ranging in velocity from 2 to 16 cm s⁻¹, as recorded 5 cm above the sea bed in the months from May to August. Scale bars, 10 cm (sediment) and 2 cm (insert). Photograph by Thomas Pillen.

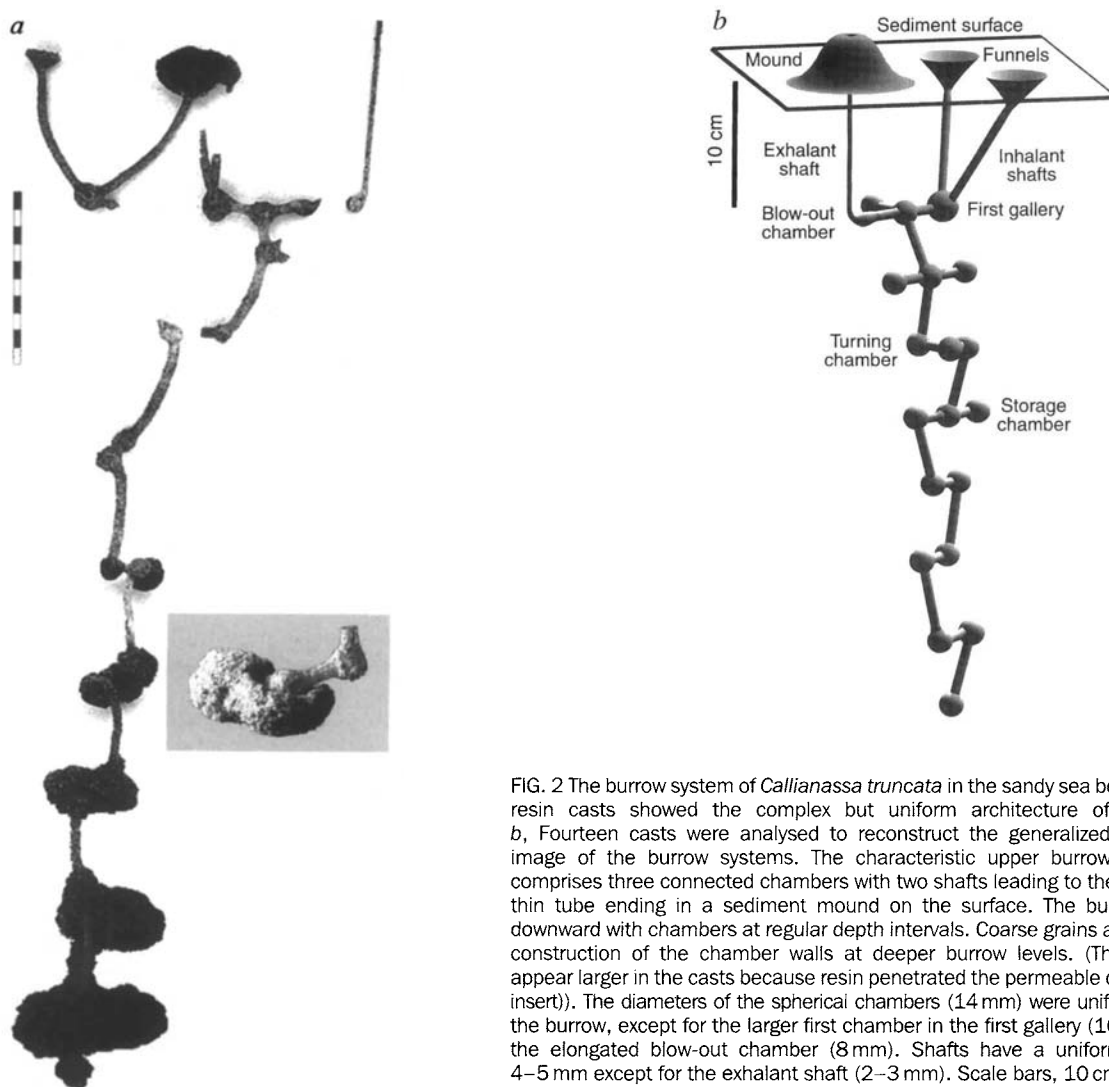


FIG. 2 The burrow system of *Callianassa truncata* in the sandy sea bed. *a*, Polyester resin casts showed the complex but uniform architecture of the burrows. *b*, Fourteen casts were analysed to reconstruct the generalized 3D computer image of the burrow systems. The characteristic upper burrow compartment comprises three connected chambers with two shafts leading to the funnels and a thin tube ending in a sediment mound on the surface. The burrow continues downward with chambers at regular depth intervals. Coarse grains are used for the construction of the chamber walls at deeper burrow levels. (These chambers appear larger in the casts because resin penetrated the permeable coarse walls (*a*, insert)). The diameters of the spherical chambers (14 mm) were uniform throughout the burrow, except for the larger first chamber in the first gallery (16–17 mm) and the elongated blow-out chamber (8 mm). Shafts have a uniform diameter of 4–5 mm except for the exhalant shaft (2–3 mm). Scale bars, 10 cm.

when passing over a mound but is retarded in the upstream and downstream regions. The resulting lateral velocity gradients cause pressure differences that drive advective pore-water flows^{12–14}. In the high-pressure areas, oxygen-rich supernatant water is forced into the permeable sediment and flows in a curved path towards the lee side of the mound where anoxic pore water emerges because of the lower pressure. Pore-water solutes, such as nutrients or metal ions are thereby released into the water column. The local advective solute transport by far exceeds the molecular diffusion across the sediment–water interface.

The flow pattern and the resulting oxygen distribution was studied around a *Callianassa* mound that had been established in sediment cores from the natural habitat and placed within a laboratory flow channel (Fig. 3). Oxygen was transported roughly 40 mm by the advective pore-water flow, whereas oxygen penetration at a smooth surface did not exceed 4 mm. In the high-pressure areas, upstream and downstream of the mound, high oxygen concentrations ($>100\ \mu\text{M}$) persisted to a sediment depth of 20 mm. The sediment volume adjacent to a mound of height 1 cm that was supplied with oxygen under a current of $10\ \text{cm s}^{-1}$ was calculated to be $90\ \text{cm}^3$ and thereby increased locally 4.5-fold compared to the oxic zone underneath a flat surface. The availability of oxygen for aerobic degradation processes was thus enhanced in the flume sediment with 22 mounds per m^2 by a factor of 1.5. This explained a 1.7-fold increase in total oxygen uptake of this sediment measured in the water column of a gas-tight sealed flume compared to a smooth sediment in a parallel

set-up. The lateral velocity gradients of the bottom-water flow also caused a passive ventilation^{14,15} of the upper part of the burrow system. Because of the low pressure, burrow water was sucked out through the exhalant opening on top of the mound. A compensating inflow of oxygen-rich water occurred through the funnel openings. The hydrodynamically induced water flow had a velocity of $0.3\text{--}0.4\ \text{cm s}^{-1}$ in the inhalant shafts and about twice that in the exhalant tube. Organic material, mainly sea-grass debris, was physically trapped in the funnels. The shrimps either feed directly on the organic material and the associated bacteria or store it in blind chambers of the burrow system for further microbial decomposition. This active downward transport provided a fast burial of organic material in the sea bed.

In addition to the hydrodynamically induced flushing, the shrimps produce a water current through their burrow system by a regular beating of their pleopods. Until now it was not known how deep oxygen was transported into the burrows and how far it penetrated into the surrounding sediment and thus how it affected the geochemistry. *In situ* studies were therefore done. A hexagonal acrylic observatory, large enough to accommodate one or two SCUBA divers, was buried 1.2-m deep in the sand with its lid level with the sediment surface. Two months after deployment, the surrounding sediment had been reinhabited by *C. truncata* at natural densities. Silicone-filled holes in the acrylic walls allowed direct sampling and measurements in intact burrows. Oxygen microelectrodes¹⁶ were inserted horizontally into the burrows and the surrounding sediment using micromanipulators. Contin-

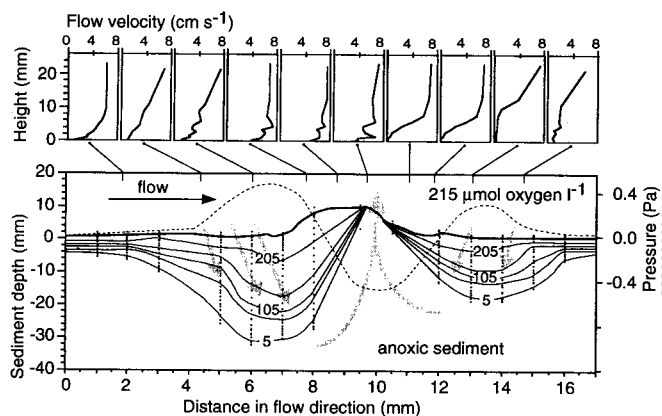


FIG. 3 Water flow and oxygen distribution around a 1-cm high *Callianassa* mound. Upper graph: flow velocity profiles; lower graph: oxygen (isopleths) and hydrodynamic pressure distribution. Measurements were taken in a laboratory flow channel (200 × 30 × 12 cm) containing a natural sediment core (60 × 30 × 20 cm; median grain size, 350 μm , permeability k (defined as a material constant related to the pore size of a sediment by the tortuosity factor²⁰) = $5.1 \times 10^{-11} \text{ m}^2$; porosity, 0.4) inhabited by *C. truncata*. The surface of the sediment core was level with the channel floor. Flow velocities were measured at 1 mm vertical resolution using a thermistor probe¹⁸. Fourteen oxygen profiles (vertical dotted lines) were recorded using Clark-type microelectrodes¹⁵ driven into the sediment at 250 μm depth increments. The isopleths connect points of equal oxygen concentration at 50 μM intervals. Fifteen pressure ports were located at the sediment–water interface and connected to a differential pressure gauge by which the pressure distribution over the mound was measured. Grey arrows indicate the flow paths of water and pore fluid. Oxygen penetrated down to 38 mm, whereas oxygen penetration did not exceed 4 mm in the area not affected by the mound.

uous oxygen measurements at 26 cm and 48 cm sediment depth revealed that *C. truncata* maintained burrow-water oxygen concentrations at 10–40% and 3–12% of air saturation, respectively, at the two depths (Fig. 4a). Horizontal oxygen microelectrode profiles measured in steps of 250 μm perpendicular to the burrow showed that oxygen penetrated 6–7 mm through the highly permeable chamber walls into the ambient sediment, whereas oxygen penetration out from the vertical shafts was only 3 mm at both depths (Fig. 4b, c). An oxic sediment environment of 12–25-mm diameter thus surrounds each burrow. Given the population density of 120 individuals per m^2 , each 10 cm × 10 cm area of the seabed thus contained one complex structure, some centimetres wide, of oxic sediment penetrating down at least half a metre.

By the active exchange of burrow water, products of organic

degradation, such as ammonium, are efficiently transported into the overlying water. The flushing with oxygenated seawater also enhances the bacterial ammonium oxidation and probably other bacterial activities in the sediment¹⁷. Ammonium concentrations measured inside the burrows were thus low (2–14 $\mu\text{mol l}^{-1}$) down to a depth of 60 cm. In the pore water of the surrounding sediment, ammonium was depleted to <15 $\mu\text{mol l}^{-1}$ at a distance of up to 3–4 cm as compared with 100 $\mu\text{mol l}^{-1}$ in surrounding sediment not inhabited by *C. truncata*. The depletion affects a sediment column of roughly 10 cm diameter around each burrow. At the observed abundance, the ammonium concentration was thus kept low in the entire sediment down to at least 50-cm depth (Fig. 4). The oxygenation of the sediment caused by the bioirrigation by *C. truncata* was also apparent from the marked differences

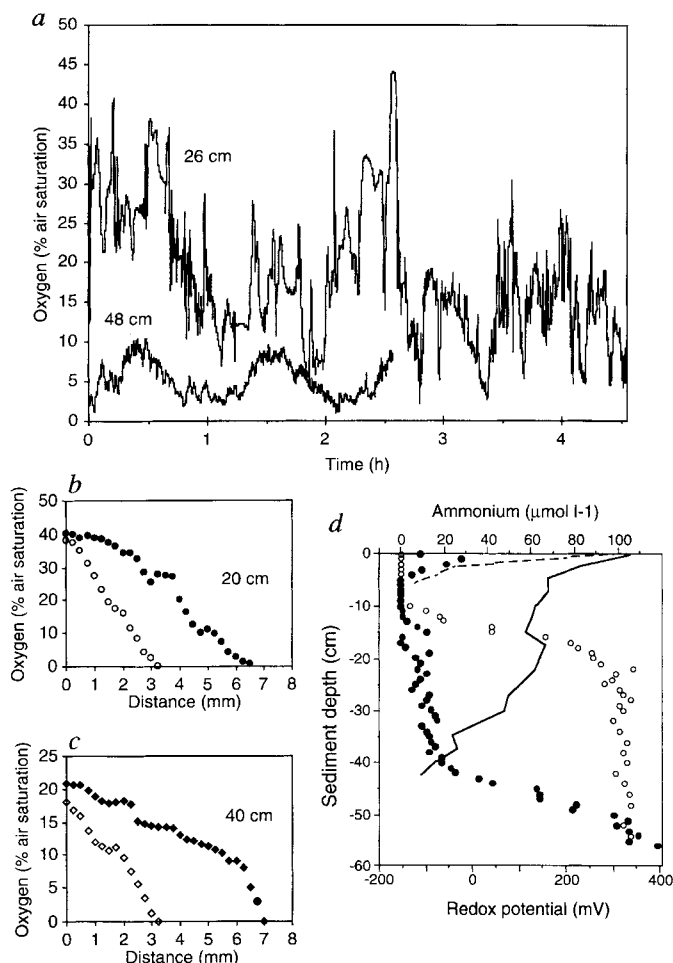


FIG. 4 a, Continuous oxygen measurements within a burrow system at 26 cm and 48 cm sediment depth. b, c, Oxygen micro-profiles measured at roughly 20 and 40 cm sediment depths from inside the burrow and out through chamber walls (●, ◆) and tube walls (○, □) into the surrounding sediment. These measurements were made *in-situ* from a hexagonal observatory (1.2 m high, 2 m diameter) deployed in the sea bed at 5-m water depth. Six acrylic walls allowed direct observations and measurements through silicone-filled holes. Continuous oxygen data were recorded by a 12-bit data logger. The instruments were self-contained in water-tight housings. One electrode was inserted into the burrow lumen and a second electrode was introduced into the ambient sediment at the same depth. This control showed that oxygen did not penetrate through the silicone ports and that measurements inside the burrows thus represented true *in situ* concentrations. d, Redox and ammonium profiles in sediment inhabited (— and ●) by *C. truncata* compared to profiles in a non-inhabited (--- and ○) sediment. The latter was established in cages, with 1-mm mesh size to exclude the macrofauna, which were attached to the wall outside the observatory. Ammonium samples were taken with evacuated serum vials closed with silicone stoppers. Using two connected syringes with a filter (pore size, 45 μm) in between, small samples (1 ml) were sucked into the vial by inserting one end into the burrow or sediment through the silicone ports in the observatory walls, while the other end penetrated through the silicone stopper into the vial. Samples were analysed within 24 h by the flow-injection method modified for small samples¹⁹. Redox potentials in the bulk sediment were measured using platinum electrodes inserted through silicone stoppers in the acrylic glass walls at vertical steps of 1 cm down to 56-cm sediment depth.

in redox potential existing between inhabited and non-inhabited sediment (Fig. 4d).

The *in-situ* studies from our sediment observatory revealed that the mud shrimp, *C. truncata*, although only 2-cm long, constructs unique burrows of extreme architectural and functional complexity that influence the whole sedimentology and geochemistry of the sea bed. The design of the upper burrow system, including mounds, funnels and first gallery, induces a hydrodynamic water exchange in the upper sediment layer. The biopumping of dissolved oxygen down to 60–80 cm sediment depth and the transport of detrital particles to similar depths can strongly accelerate the degradation of organic matter in the sandy sediments. □

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1. Berner, R. A. *Early Diagenesis: A Theoretical Approach* (Princeton Univ. Press, Princeton, 1980).
2. Revsbech, N. P., Jørgensen, B. B. & Blackburn, P. H. *Science* **207**, 1355–1356 (1980).
3. Aller, R. C. in *Animal–Sediment Relations* (eds McCall, P. L. & Tevesz, M. J. S.) 53–104 (Plenum, London, 1982).
4. Boudreau, B. P. *Geochim. Cosmochim. Acta* **58**, 1243–1249 (1994).

5. Aller, R. C. & Dodge, R. E. *J. Mar. Res.* **32**, 209–232 (1974).
6. Griffis, R. B. & Suchanek, T. H. *Mar. Ecol. Prog. Ser.* **79**, 171–183 (1991).
7. Dworschak, P. C. *Mar. Ecol. Prog. Ser.* **4**, 19–43 (1983).
8. Pemberton, S. G., Risk, M. J. & Buckley, D. E. *Science* **192**, 790–791 (1976).
9. Forster, S. & Graf, G. *Mar. Biol.* **123**, 335–346 (1995).
10. Ray, A. J. & Aller, R. C. *Mar. Geol.* **62**, 371–379 (1985).
11. Schlichting, H. *Boundary Layer Theory* 7th edn (McGraw-Hill, New York, 1987).
12. Huettel, M. & Gust, G. *Mar. Ecol. Prog. Ser.* **89**, 253–267 (1992).
13. Yager, P. L., Nowell, R. A. M. & Jumars, P. A. *J. Mar. Res.* **51**, 209–236 (1993).
14. Vogel, S. *Life in Moving Fluids* (Princeton Univ. Press, New Jersey, 1983).
15. Allanson, B. R., Skinner, D. & Imberger, J. *Est. Coast. Shelf Sci.* **35**, 253–266 (1992).
16. Revsbech, N. P. *Limnol. Oceanogr.* **34**, 474–478 (1989).
17. Kristensen, E. J. *Coast. Res.* **1**, 109–116 (1985).
18. LaBarbera, M. & Vogel, S. *Limnol. Oceanogr.* **21**, 750–756 (1976).
19. Hall, P. O. J. & Aller, R. C. *Limnol. Oceanogr.* **37**, 1113–1119 (1992).
20. Hsu, K. J. *Physical Principles of Sedimentology* (Springer, Berlin, Heidelberg, 1989).

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CORRESPONDENCE and requests for materials should be addressed to W.Z. (e-mail: wiebke@postgate.mmi-mm.uni-bremen.de).

Genetically lean mice result from targeted disruption of the RII β subunit of protein kinase A

David E. Cummings, Eugene P. Brandon, Josep V. Planas, Kouros Motamed, Rejean L. Idzerda & G. Stanley McKnight

Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington 98195–7750, USA

CYCLIC AMP is an important second messenger in the coordinated regulation of cellular metabolism. Its effects are mediated by cAMP-dependent protein kinase (PKA), which is assembled from two regulatory (R) and two catalytic (C) subunits. In mice there are four R genes (encoding RIA, RI β , RIIa, and RII β) and two C genes (encoding Ca and C β), expressed in tissue-specific patterns¹. The RII β isoform is abundant in brown and white adipose tissue and brain, with limited expression elsewhere. To elucidate its functions, we generated RII β knockout mice. Here we report that mutants appear healthy but have markedly diminished white adipose tissue despite normal food intake. They are protected against developing diet-induced obesity and fatty livers. Mutant brown adipose tissue exhibits a compensatory increase in RIIa,

which almost entirely replaces lost RII β , generating an isoform switch. The holoenzyme from mutant adipose tissue binds cAMP more avidly and is more easily activated than wild-type enzyme. This causes induction of uncoupling protein and elevations of metabolic rate and body temperature, contributing to the lean phenotype. Our results demonstrate a role for the RII β holoenzyme in regulating energy balance and adiposity.

RII β null mutant mice were generated by targeted gene disruption; details of the knockout construction will be published elsewhere. Western blotting confirmed the absence of RII β protein in homozygous mutants. RII β mutants are fertile and long lived, exhibiting no overt abnormal phenotype. However, they have remarkably decreased white adipose tissue (WAT) mass. The weights of fat pads from three anatomical locations in mutants were about half those of the wild types (Table 1). Magnetic resonance imaging (Fig. 1a) confirmed that the reduction in fat occurs throughout the whole body. Whole-mouse magnetic resonance spectroscopy² and volume-of-distribution experiments with ³H₂O showed that mutants have approximately 6% body fat, compared with 15% in the wild type.

The knockout mice are not cachectic. Overall weight is reduced by suggesting ~10%, that loss of fat is probably the only alteration in body composition (Table 1). Leanness does not seem to result from decreased food intake or absorption. Mutants tend to be slightly hyperphagic (Table 1), and show normal postprandial triglyceride blood levels. Plasma cholesterol, free fatty acids, insulin, glucose and thyroid hormones are also unperturbed (data not shown). The reduction of WAT seems to arise principally from decreased triglyceride stores, rather than diminished

TABLE 1 Knockout mice have reduced adipose tissue mass

	Reproductive fat-pad weight (% body weight)	Inguinal fat-pad weight (% body weight)	Retroperitoneal fat-pad weight (% body weight)	Reproductive fat-pad cellularity ($\times 10^6$ cells per pad)	Inguinal fat-pad cellularity ($\times 10^6$ cells per pad)	Retroperitoneal fat-pad cellularity ($\times 10^6$ cells per pad)	Body weight (g)	Food intake (kcal per mouse per d)
Male								
Wild type	1.29 $\pm$ 0.10	0.96 $\pm$ 0.10	0.29 $\pm$ 0.04	17.4 $\pm$ 0.9	18.6 $\pm$ 1.9	3.7 $\pm$ 0.5	25.2 $\pm$ 0.9	11.4 $\pm$ 2.7
Mutant	0.62 $\pm$ 0.04	0.63 $\pm$ 0.04	0.11 $\pm$ 0.01	15.4 $\pm$ 1.5	21.4 $\pm$ 2.6	3.4 $\pm$ 0.5	23.4 $\pm$ 1.1	12.4 $\pm$ 1.5
% of wild type	48%	65%	37%	89%	115%	92%	93%	109%
	$P \leq 0.0001$	$P \leq 0.002$	$P \leq 0.0001$	$P = 0.14$	$P = 0.21$	$P = 0.31$	$P = 0.10$	$P = 0.39$
Female								
Wild type	2.25 $\pm$ 0.34	1.09 $\pm$ 0.03	0.23 $\pm$ 0.02	19.0 $\pm$ 2.0	21.6 $\pm$ 2.3	ND	22.4 $\pm$ 1.7	9.1 $\pm$ 0.4
Mutant	0.97 $\pm$ 0.13	0.72 $\pm$ 0.04	0.11 $\pm$ 0.02	17.4 $\pm$ 1.7	21.1 $\pm$ 2.8	ND	19.5 $\pm$ 0.8	10.9 $\pm$ 0.3
% of wild type	43%	66%	48%	92%	98%		87%	120%
	$P \leq 0.0006$	$P \leq 0.0001$	$P \leq 0.001$	$P = 0.21$	$P = 0.44$		$P = 0.06$	$P = 0.01$

RII β null mutants have reduced adipose tissue mass despite adequate food intake. Discrete white fat pads from three anatomical sites were dissected and weighed. 'Reproductive' refers to the epididymal and parametrial pads of males and females, respectively. White adipose tissue cellularity was ascertained by DNA quantitation. Consumption of standard mouse chow (Harlan Teklad) was measured over four months starting at six weeks of age. Results are means $\pm$ s.e.m.

adipocyte number, as revealed by the normal cellularity of fat pads (Table 1) and reduced adipocyte size (Fig. 1*b*). There are no obvious histological changes in tissues other than fat.

Mutants are protected against some of the adverse effects of consuming a high-fat diet. After eating a 58% fat diet for four months, normal mice became obese, whereas mutants remained lean (Fig. 1*d*). Furthermore, wild types developed fatty livers but mutants did not (Fig. 1*e, f*).

Mutants seem to be lean, at least in part because of changes in PKA activity and gene expression in brown adipose tissue (BAT). BAT facilitates non-shivering thermogenesis during cold acclimation³ or chronic overeating⁴. This is accomplished by uncoupling protein (UCP), a proton translocator that uncouples mitochondrial respiration from oxidative phosphorylation in BAT, so that energy derived from oxidation of fatty acids is dissipated as heat, rather than stored as ATP. UCP is induced by a cAMP-dependent mechanism after β -adrenergic stimulation⁵.

RII β is the principal PKA regulatory subunit in BAT. It is expressed more abundantly there than in any other tissue (data not shown). Although there are no histological changes in mutant BAT, loss of RII β protein is associated with a compensatory increase of the RI α isoform, normally scarce in this tissue (Fig.

2*a*). RI β is not expressed in BAT, and RII α is barely detectable in both wild type and mutants. Thus, elimination of RII β transforms the PKA in BAT from a predominantly RII-containing (type II) holoenzyme to a predominantly RI type. High-performance liquid chromatography (HPLC) separation of type I and II PKA confirms this isoform switch (Fig. 2*b*). The increased RI α compensates almost entirely for lost RII β , such that overall cAMP-binding capacity (a reflection of total R subunit quantity) is reduced by only $\sim 10\%$ (Fig. 3*a*). Total C subunit is decreased by roughly one third as measured by kinase activity (Fig. 3*b*) and western analysis (Fig. 2*a*). Thus it seems that an R:C ratio of at least 1:1 is maintained in mutant BAT. These changes in RI α and C α arise by post-transcriptional mechanisms (P. S. Amieux and G.S.M., unpublished results).

In BAT, the largely type-I PKA in mutants binds cAMP more avidly than does the type-II PKA predominating in the wild type, with K_d values of 140 and 380 nM, respectively (Fig. 3*a*). Mutant PKA holoenzyme is consequently more readily activated by cAMP than is wild-type enzyme (K_a values of 80 and 350 nM, respectively, Fig. 3*b*). Accordingly, basal PKA activity is elevated by roughly five times in mutant BAT (Fig. 3*b*, insert). cAMP levels in wild-type and mutant BAT showed no differences that might be

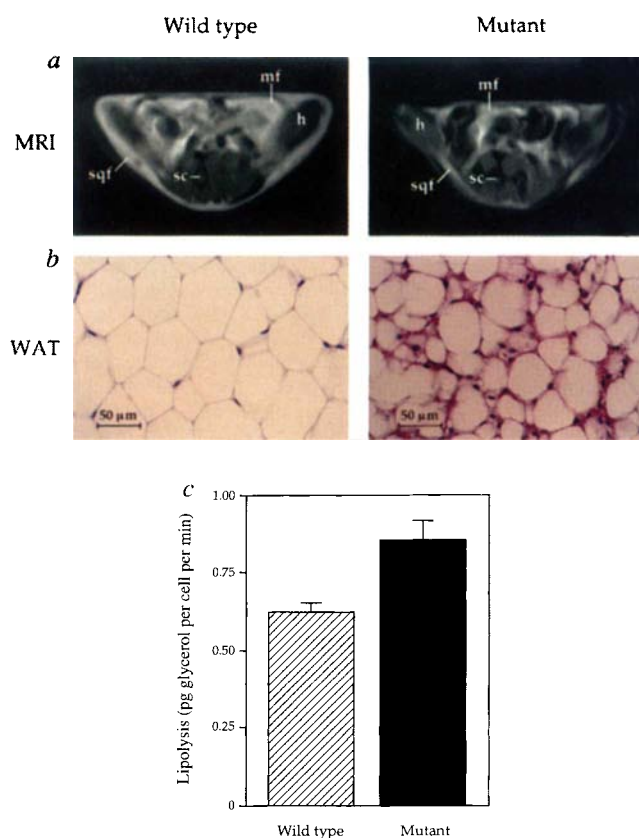
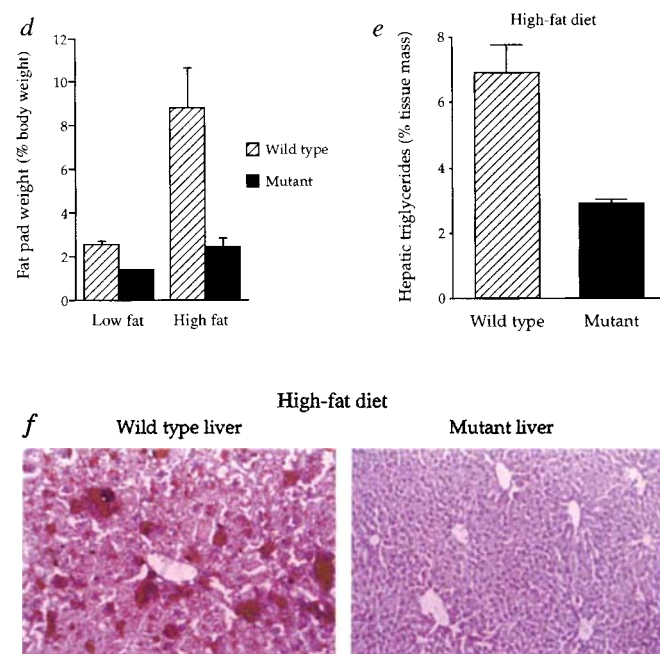


FIG. 1 RII β mutants have reduced fat throughout their bodies and resist diet-induced obesity. *a*, Fat-selective magnetic resonance images of female mice. Spin echo inversion-recovery sequences weighted by the magnetic relaxation time constant T1 are shown, in which only adipose tissue appears white. Images are 3-mm thick axial sections at the base of the hind legs. Similar results were obtained throughout the body. Abbreviations: mf, mesenteric fat; sqf, subcutaneous fat; sc, spinal cord; h, proximal hind leg. *b*, Representative histology of normal WAT, composed of large, polygonal cells with prominent triglyceride deposits (clear spaces) and flattened nuclei. Comparable RII β mutant WAT reveals smaller, rounded adipocytes with diminished triglyceride stores, more eosinophilic aqueous cytoplasm, plumper nuclei, and scattered multivesicular cells. Sections were formalin fixed, paraffin embedded, and stained with haematoxylin and eosin. *c*, RII β mutant WAT has increased lipolysis, as assessed by glycerol release.



Washed epididymal fat-pad fragments were gently shaken at 37 °C in Krebs Ringer-HEPES buffer (pH 7.2) with 1% bovine serum albumin. Glycerol content of the medium was determined at six time points over two hours using an enzymatic assay (Sigma); cell number was assessed by DNA quantification. Results are means $\pm$ s.e.m. from five pairs of mice ($P = 0.01$). *d*, RII β mutants resist diet-induced obesity. Combined weights of bilateral epididymal, inguinal and retroperitoneal fat pads from 6 mice per group were measured after four months on either an 11% or 58% lipid diet²⁵. On the high-fat diet normal mice developed markedly increased adiposity, whereas mutants remained lean. Overall body weights of both groups were only slightly increased. *e*, *f*, Mutants are protected from developing fatty livers on the high-fat diet. Hepatic triglycerides were quantified in five wild-type and five mutant mice with an enzymatic assay (Boehringer Mannheim). Values are means $\pm$ s.e.m. ($P = 0.001$). Haematoxylin and eosin-stained liver sections were counterstained with oil-red-O to reveal abnormal lipid deposits (bright red) in hepatocytes of normal animals. This pathology is absent in mutants. Specialized mouse food was provided by Research Diets, Inc. By calories, the low-fat diet contained 60% corn starch and 7% hydrogenated coconut oil, whereas the high-fat diet contained 13% corn starch and 54% coconut oil. Both diets contained 12% maltodextrin, 4% soybean oil, 16% casein, and identical vitamins and minerals.

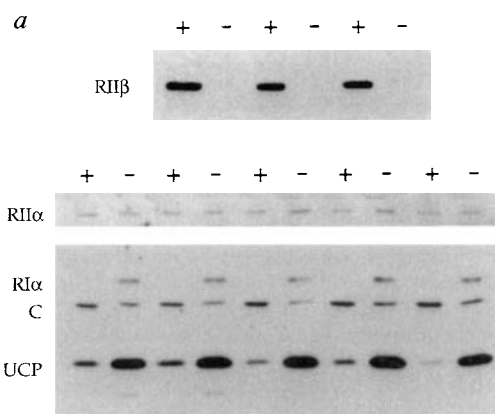
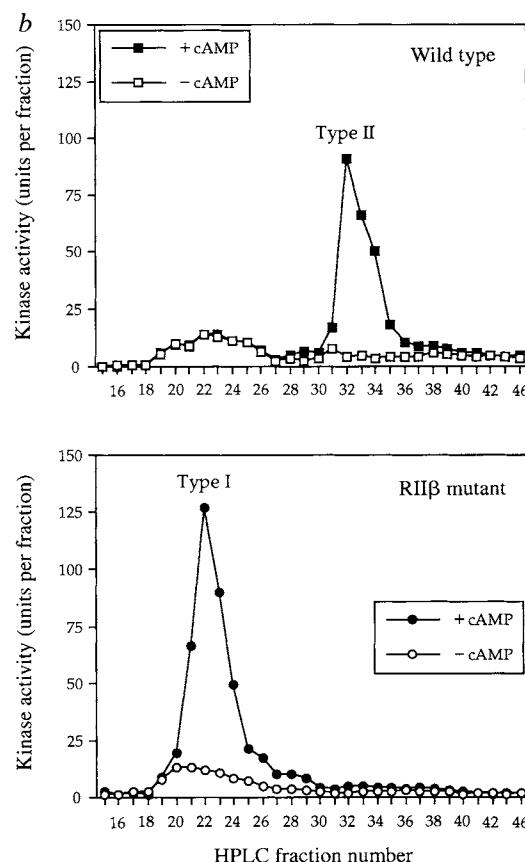


FIG. 2 a, Changes in protein levels and PKA activity in RII β mutant BAT. Western blots of BAT from wild-type (+) and knockout (-) mice were probed with the antibodies shown. Elimination of RII β in mutants is confirmed by the complete absence of RII β protein in BAT. The RII α isoform is markedly increased in mutants, whereas there is a small loss of C subunit. RII β is not detectable in either wild-type or mutant adipose tissue. RII α is barely detectable, and is unaltered in mutants. UCP is increased 4–5-fold in mutants, as revealed by scanning densitometry. **b**, Mutant BAT displays an isoform switch from type II to type I PKA. Type I (fractions 20–25) and type II (fractions 31–35) PKA from BAT homogenates were resolved by ion exchange chromatography. Displayed are basal (–cAMP) and total activatable (+cAMP) kinase activity.

METHODS. BAT protein homogenates (40 μ g per lane) were resolved by 10% SDS–PAGE and transferred to nitrocellulose. Coomassie Blue staining confirmed uniformity of the protein loading. Immunoblotting was performed with polyclonal antisera raised against recombinant murine PKA subunits and diluted as follows: RII β 1:10,000; RII α 1:1,000; RI 1:200; and C 1:2,000. The anti-RI antisera recognizes both RII α and RII β , which are resolvable using this gel system; the anti-C antibody recognizes both C α and C β , which co-migrate. Anti-RII β and RII α antisera were provided by J. Scott, and anti-hamster UCP (1:2,000) by L. Kozak. Primary antibodies were visualized with horseradish peroxidase-coupled goat anti-rabbit Ig (1:40,000) and ECL (Amersham). Ion exchange chromatography was performed as previously described²⁶. BAT protein (4 mg) was loaded onto



DEAE columns and separated by HPLC with a linear salt gradient from 0 to 250 mM NaCl. Kinase activity of individual fractions was determined with Kemptide substrate as previously described²⁷, in the presence or absence of 5 μ M cAMP. Type I PKA elutes between 40 and 80 mM NaCl (fractions 20–25), and type II between 130 and 180 mM NaCl (fractions 31–35). Free C subunit elutes just before type I holoenzyme.

attributed to extrinsic (for example, sympathetic) stimulation (data not shown). These data suggest that the changes in PKA activity result from the type II to type I isoform switch, rather than from a gross deregulation of C subunits due to either loss of R subunits or increased cAMP.

The elevated basal PKA activity leads to a four- to fivefold increase in UCP (Fig. 2a), at least partly by inducing UCP messenger RNA (data not shown). Figure 3c, d shows that knockouts have an elevated metabolic rate (assessed by oxygen consumption, which closely reflects energy expenditure⁶), and a 0.8 °C increase in body temperature (Fig. 3e). These findings suggest that excess UCP in BAT, arising from increased basal PKA tone, renders RII β -mutant mice metabolically inefficient, causing food calories to be wasted as heat and depleting fat stores. Furthermore, lipoprotein lipase mRNA in BAT (whose expression is also PKA dependent⁷) seems to be overexpressed, possibly helping mutant BAT to compete with WAT for dietary triglycerides, to be burned rather than stored.

After BAT, RII β is most highly expressed in WAT, where it is also the principal PKA regulatory isoform (data not shown). In this tissue, PKA transduces signals from catecholamines and glucagon to stimulate lipolysis and inhibit lipogenesis. Like BAT, RII β mutant WAT displays increased RII α , a type II to I isoform switch, and elevated basal PKA activity despite moderately decreased total PKA activity and C-subunit protein (data not shown). These changes are associated with a 35% increase in basal lipolysis in cultured adipocytes (Fig. 1c), which may contribute to the lean phenotype. However, serum glycerol and free fatty acids were similar in wild-type and mutant mice. As both BAT and

WAT are affected in RII β mutants, we are unable to determine their relative contributions to the lean phenotype without generating a tissue-specific RII β defect.

Important advances in understanding body-weight regulation have resulted from the study of genetically obese rodent strains^{8–12}, of which there are at least 12 (refs 13–15). RII β mutants are one of only a few animal models of genetic leanness^{16–18}. Several obese rodent strains have reduced UCP and thermogenically inactive BAT, in contrast to RII β mutants³. It will be interesting to see if the RII β knockout can rescue an obese phenotype when two mutant strains are interbred.

RII β mutants have markedly reduced leptin mRNA and plasma levels (data not shown), corroborating the proposed role of leptin as an adiposity indicator⁸. Leptin is also thought to be a satiety factor^{19–21}. However, only mild hyperphagia, insufficient to maintain normal adiposity, is seen in RII β mutants despite reduced leptin levels. A change in the mutants' body-weight regulatory system could exist, preventing them from developing fully compensatory hyperphagia. It has been proposed that BAT might produce an appetite-suppressing signal²², because of the unexpected finding that BAT-deficient mice develop hyperphagia¹⁴. It is possible that such a factor is induced in the overactive BAT of RII β mutants, inhibiting them from eating enough to maintain normal lipid stores.

One way that the brain is thought to regulate adiposity is by modulating sympathetic stimulation of PKA in BAT to control facultative energy expenditure through UCP³. RII β mutants provide evidence for this phenomenon, as their elevated basal PKA activity in BAT is associated with increased UCP, excess

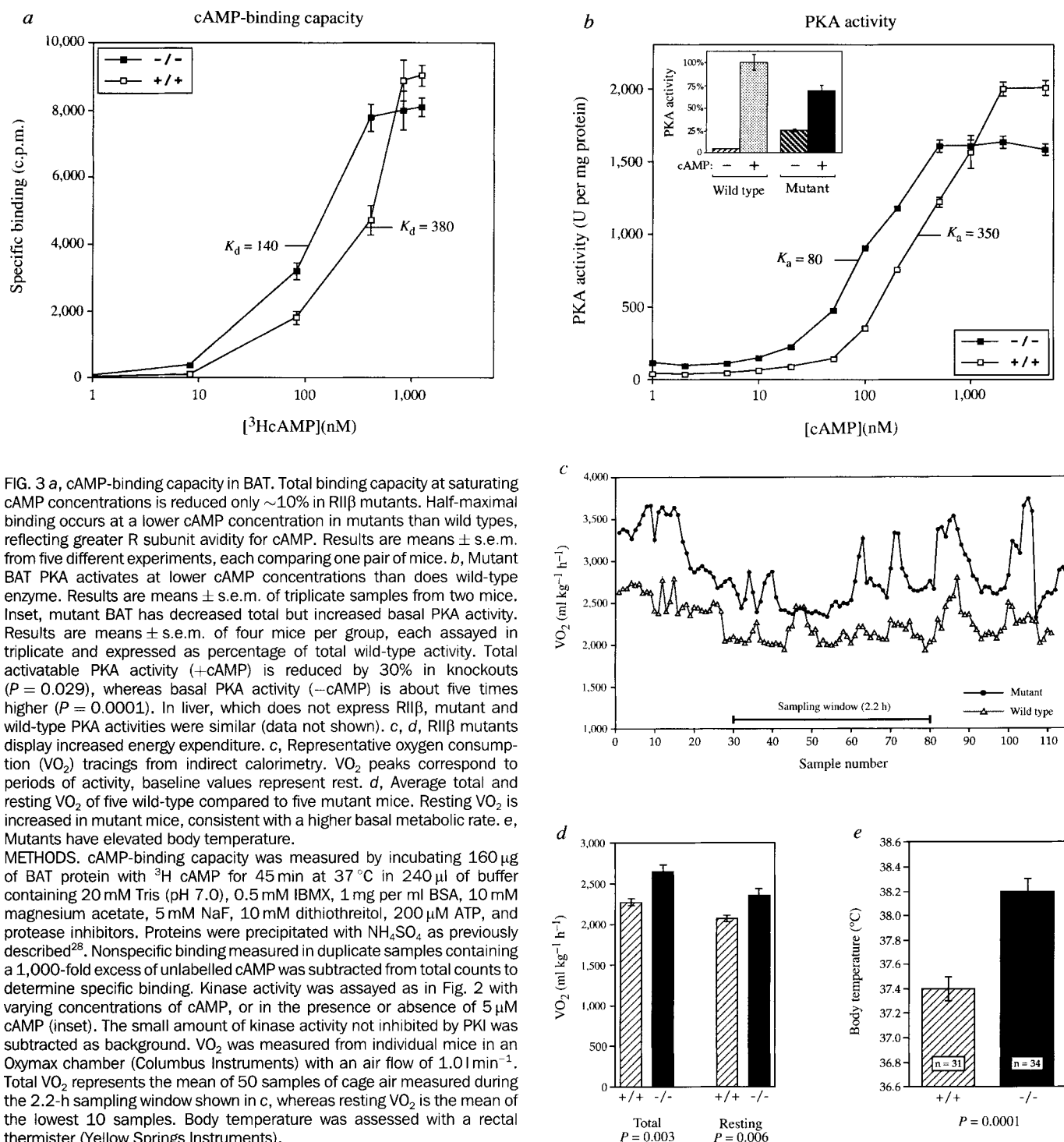


FIG. 3 **a**, cAMP-binding capacity in BAT. Total binding capacity at saturating cAMP concentrations is reduced only $\sim 10\%$ in RII β mutants. Half-maximal binding occurs at a lower cAMP concentration in mutants than wild types, reflecting greater R subunit avidity for cAMP. Results are means $\pm$ s.e.m. from five different experiments, each comparing one pair of mice. **b**, Mutant BAT PKA activates at lower cAMP concentrations than does wild-type enzyme. Results are means $\pm$ s.e.m. of triplicate samples from two mice. Inset, mutant BAT has decreased total but increased basal PKA activity. Results are means $\pm$ s.e.m. of four mice per group, each assayed in triplicate and expressed as percentage of total wild-type activity. Total activatable PKA activity (+cAMP) is reduced by 30% in knockouts ($P = 0.029$), whereas basal PKA activity ($-$ cAMP) is about five times higher ($P = 0.0001$). In liver, which does not express RII β , mutant and wild-type PKA activities were similar (data not shown). **c**, **d**, RII β mutants display increased energy expenditure. **c**, Representative oxygen consumption (VO_2) tracings from indirect calorimetry. VO_2 peaks correspond to periods of activity, baseline values represent rest. **d**, Average total and resting VO_2 of five wild-type compared to five mutant mice. Resting VO_2 is increased in mutant mice, consistent with a higher basal metabolic rate. **e**, Mutants have elevated body temperature.

METHODS. cAMP-binding capacity was measured by incubating $160 \mu\text{g}$ of BAT protein with ^3H cAMP for 45 min at 37°C in $240 \mu\text{l}$ of buffer containing 20 mM Tris (pH 7.0), 0.5 mM IBMX, 1 mg per ml BSA, 10 mM magnesium acetate, 5 mM NaF, 10 mM dithiothreitol, 200 μM ATP, and protease inhibitors. Proteins were precipitated with NH_4SO_4 as previously described²⁸. Nonspecific binding measured in duplicate samples containing a 1,000-fold excess of unlabelled cAMP was subtracted from total counts to determine specific binding. Kinase activity was assayed as in Fig. 2 with varying concentrations of cAMP, or in the presence or absence of $5 \mu\text{M}$ cAMP (inset). The small amount of kinase activity not inhibited by PKI was subtracted as background. VO_2 was measured from individual mice in an Oxygraph chamber (Columbus Instruments) with an air flow of 1.0 l min^{-1} . Total VO_2 represents the mean of 50 samples of cage air measured during the 2.2-h sampling window shown in **c**, whereas resting VO_2 is the mean of the lowest 10 samples. Body temperature was assessed with a rectal thermister (Yellow Springs Instruments).

energy expenditure, and leanness. In contrast, mice made deficient in BAT with a toxigenic transgene develop an abnormally efficient metabolism and obesity¹⁴. Chronic sympathetic stimulation normally causes BAT hypertrophy²³. However, wild-type and mutant interscapular BAT showed no differences in weight (65 ± 17 compared to 60 ± 17 mg, respectively) or DNA content (119 ± 7 compared to $123 \pm 5 \mu\text{g}$), and mutant tissue had only slightly increased protein content ($8.8 \pm .5$ compared to $10.9 \pm 1.2 \text{ mg}$). In mice, adrenergic stimulation of β_1 receptors is felt to be the principal mediator of BAT hypertrophy, whereas β_3 receptors are probably most important for UCP induction²³. PKA changes in RII β mutant BAT mimic β_3 more than β_1 stimulation, because they do not cause hypertrophy.

Chronic activation of PKA in adipose tissue through β -adrenergic stimulation is being investigated as a mode of obesity therapy. Research has focused on agonists specific for β_3 receptors²⁴, which are adipose specific²³. Our results raise the possibility that RII β could also provide a target for anti-obesity drugs. $\square$

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- McKnight, G. S. *Curr. Opin. Cell Biol.* **3**, 213–217 (1991).
- Stein, D. T., Babcock, E. E., Malloy, C. R. & McGarry, J. D. *Int. J. Obes. relat. metab. Disord.* **19**, 804–810 (1995).
- Himmels-Hagen, J. *FASEB J.* **4**, 2890–2898 (1990).
- Rothwell, N. J. & Stock, M. J. *Nature* **281**, 31–35 (1979).
- Kopecky, J. et al. *J. Biol. Chem.* **265**, 22204–22209 (1990).
- Jequier, E. & Felber, J.-P. *Bailliere's clin. Endocr. Metab.* **1**, 911–935 (1987).
- Carneheim, C., Nedergaard, J. & Cannon, B. *Am. J. Physiol.* **254**, E155–E161 (1988).

8. Zhang, Y. et al. *Nature* **372**, 425–432 (1994).
9. Chen, H. et al. *Cell* **84**, 491–495 (1996).
10. Lee, G.-H. et al. *Nature* **379**, 632–635 (1996).
11. Chua, S. C. et al. *Science* **271**, 994–997 (1996).
12. Noben-Trauth, K., Naggert, J. K., North, M. A. & Nishina, P. M. *Nature* **380**, 534–538 (1996).
13. Friedman, J. M. & Leibel, R. L. *Cell* **96**, 217–220 (1992).
14. Lowell, B. B. et al. *Nature* **366**, 740–742 (1993).
15. Tecott, L. H. et al. *Nature* **374**, 542–546 (1995).
16. Schneider, A., Davidson, J.J., Wulrich, A. & Kilmann, M. W. *Nature Genet.* **5**, 381–385 (1993).
17. Katz, E. B., Stenbit, A. E., Hatton, K., DePinho, R. & Charron, M. J. *Nature* **377**, 151–155 (1995).
18. Kozak, L. P., Kozak, U. C. & Clarke, G. T. *Genes Dev.* **5**, 2256–2264 (1991).
19. Pelleymounter, M. A. et al. *Science* **269**, 540–543 (1995).
20. Halaas, J. L. et al. *Science* **269**, 543–546 (1995).
21. Campfield, L. A., Smith, F. J., Guise, Y., Devos, R. & Burn, P. *Science* **269**, 546–549 (1995).
22. Flier, J. S. *Cell* **80**, 15–18 (1995).
23. Lafontan, M. & Berlan, M. J. *Lipid Res.* **34**, 1057–1091 (1993).
24. Arner, P. *New Engl. J. Med.* **333**, 382–383 (1995).
25. Surwit, R. S. et al. *Metabolism* **44**, 645–651 (1995).
26. Clegg, C. H., Cadd, G. G. & McKnight, G. S. *Proc natn. Acad. Sci. U.S.A.* **85**, 3703–3707 (1988).
27. Clegg, C. H., Correll, L. A., Cadd, G. G. & McKnight, G. S. *J. biol. Chem.* **262**, 13111–13119 (1987).
28. Doskeland, S. O. & Oegreid, D. *Meth. Enzym.* **159**, 147–150 (1988).

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CORRESPONDENCE and requests for materials should be addressed to G.S.M. (e-mail: mcknight@u.washington.edu).

Where in the brain does visual attention select the forest and the trees?

G. R. Fink*, P. W. Halligan†‡, J. C. Marshall‡, C. D. Frith*, R. S. J. Frackowiak* & R. J. Dolan*§

* Wellcome Department of Cognitive Neurology, Institute of Neurology, 12 Queen Square, London WC1N 3BG, UK

† Rivermead Rehabilitation Centre, Abingdon Road, Oxford OX1 4XD, UK

‡ Neuropsychology Unit, University Department of Clinical Neurology, The Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, UK

§ Royal Free Hospital School of Medicine, Roland Hill Street, London NW3 2PF, UK

THE perceptual world is organized hierarchically: the forest consists of trees, which in turn have leaves. Visual attention can emphasize the overall picture (global form) or the focal details of a scene (local components)¹. Neuropsychological studies have indicated that the left hemisphere is biased towards local and the right towards global processing. The underlying attentional and perceptual mechanisms are maximally impaired by unilateral lesions to the temporal and parietal cortex^{2,3}. We measured brain activity of normal subjects during two experiments using 'hierarchically' organized figures. In a directed attention task, early visual processing (prestriate) areas were activated: attention to the global aspect of the figures activated the right lingual gyrus whereas locally directed attention activated the left inferior occipital cortex. In a subsequent divided attention task, the number of target switches from local to global (and vice versa) covaried with temporal–parietal activation. The findings provide direct evidence for hemispheric specialization in global and local perception; furthermore, they indicate that temporal–parietal areas exert attentional control over the neural transformations occurring in prestriate cortex.

We measured brain activity, indexed by regional cerebral blood flow (rCBF), in two separate experiments using 'hierarchically' organized visual stimuli (Fig. 1). In the first experiment, subjects were asked to attend to and name either the global or the local aspect of the figures in separate blocks of trials. As psychophysical differences in global and local processing may be confounded by differences in stimulus size⁴, we controlled for size effects by

presenting both large and small hierarchical figures in a factorial experimental design. In the second experiment, a preselected target letter appeared at either the global or the local level and the subjects were required to say at which of the two levels the target had appeared. The number of times that the target switched from the global to the local level (or vice versa) on successive trials varied parametrically across the experiment.

In the first experiment, as predicted, marked differences in neuronal activation associated with figure size were observed in the striate (primary visual) cortex: large stimuli were associated with extensive striate activation and small stimuli with more restricted posterior (foveal) striate activations ($P < 0.05$, using statistical parametric mapping and corrected for multiple non-independent comparisons). In addition, when subjects attended to the global aspect of figures, significant increases in relative rCBF were seen in the right lingual gyrus (Brodmann area 18; $P < 0.05$, corrected; Table 1, Fig. 2). When subjects attended to the local attribute, there was a significant relative rCBF increase in the left inferior occipital cortex (BA 18; $P < 0.05$, corrected, Table 1, Fig. 2). A direct comparison of rCBF changes between the two hemispheres indicated that these lateralized effects were statistically significant ($P < 0.01$). A significant interaction between figure size and the activation due to local processing was observed in the left inferior occipital cortex (BA 18, Fig. 2) alone, where the difference

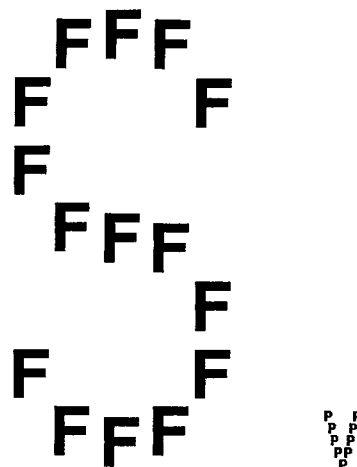


FIG. 1 Example of figures used as stimuli during the global and local processing experiments. The S and the V represent the global level and the F and the P the local level. All figures used were non-congruent for the global and local level. During the rCBF measurements, the figures were shown every 1.5 s for 300 ms in the centre of a 14 inch video display unit at a viewing distance of 40 cm. In the first experiment, 12 different letters were used to create the figures. These were presented in a quasi-random sequence that prevented the same letter appearing at the local or global level on successive trials¹⁶. To study stimulus size effects on global and local processing, both large figures (170 × 72 mm, made of letters 21 × 13 mm, visual angle subtended 24° and 10°) and small figures (30 × 14 mm, made of letters 3.5 × 2.5 mm, visual angle 4° and 2°) were used. Subjects were instructed to attend to either the global or the local level and to name the appropriate letter. rCBF measurements were performed during the following four conditions: global directed attention, large stimuli; local directed attention, large stimuli; global directed attention, small stimuli; local directed attention, small stimuli. Subjects lay with eyes open in a quiet, darkened room. Twelve rCBF measurements were performed per subject (three repeats per condition); globally and locally directed attention tasks were alternated. During the second experiment (divided attention), the same stimuli, (large letters only), presentation rate and number of measurements were used, however, this time a preselected target letter appeared at either the global or the local level. The number of switches between perceptual levels was varied on successive trials from 1 to 34 per min. The number of times that a given target letter occurred at the global or the local level was kept roughly equal across the rCBF measurements. A quasi-random sequence prevented the same stimulus from occurring on successive trials.

between local and global processing was augmented by increasing figure size. The absence of differential rCBF increases in lateral temporal-parietal areas, during either local or global tasks, led us to conjecture that this was because subjects were not required to switch attention between the two levels (blocked presentation).

FIG. 2 Relative rCBF increases (for the ten subjects) associated with globally $((A + C) - (B + D))$ and locally $((B + D) - (A + C))$ directed attention (experiment 1). Areas of significant relative rCBF increases ($P < 0.05$, corrected for multiple non-independent comparisons) are shown as through-projections onto representations of standard stereotactic space¹⁵. Sagittal, side view; transverse, view from above. Transverse SPM $\{z\}$ maps were superimposed upon the group mean magnetic resonance (MR) image, that had been spatially normalized into the same anatomical space¹⁵. Red arrows indicate the local maximum within the area of activation. There is right-hemispheric neuronal activation centred on the lingual gyrus during globally directed attention and left-hemispheric neuronal activation centred on the inferior occipital cortex during locally directed attention. R, right; VAC, vertical plane through the anterior commissure; VPC, vertical plane through the posterior commissure; numbers at axes refer to coordinates of stereotactic space¹⁵. The exact coordinates of the local maxima and their Z statistics are given in Table 1. Adjusted means rCBF (to a mean of $50 \text{ ml dl}^{-1} \text{ min}^{-1}$) $\pm$ s.e.m. for each condition are displayed for the local maxima. LG, large stimulus with global attention; LL, large with local attention; SG, small with global; SL small with local. There is a dependency on stimulus size of the adjusted blood flow response in early visual processing in both areas. A significant interaction of stimulus size on the rCBF response during local processing was observed in the left inferior occipital cortex, but no such interaction was seen in the right lingual gyrus. **METHODS.** In experiment 1, 10 normal-sighted male right-handed volunteers were studied using a position emission tomography (PET) scanner (Siemens) in 3D mode with a 15-cm axial field of view. Relative rCBF was measured from the distribution of radioactivity after slow bolus intravenous injection of H_2^{15}O (9 mCi per scan, each lasting 90 s)¹⁷. Attenuation-corrected data were reconstructed into 63 image planes with a resulting resolution of 6 mm at full-width-half-maximum. For each subject, structural images were obtained with a 2 T Magnetom VISION (Siemens). Statistical parametric mapping (SPM95) software was used for image realignment, transformation into standard stereotactic space, smoothing and statistical analysis^{18,19}. All measurements per condition were averaged across subjects. State-dependent differences in global flow were varied out using ANCOVA. Main effects and interactions were assessed with contrasts of the adjusted task means using the t-statistic subsequently transformed into the normally distributed Z statistic. The resulting set of Z values constituted a statistical parametric map (SPM $\{z\}$) which was then thresholded at $P < 0.05$ (corrected for multiple comparisons). The hemisphere $\times$ condition interaction was identified by using SPM. This did not require correction because these regions were identified on the basis of the (independent) main effects.

We addressed this hypothesis in a second experiment, in which we determined the rCBF changes that correlated with the number of attentional target switches between perceptual levels. Only activations relevant to the specific hypothesis concerning the temporal-parietal cortex and prestriate areas are reported.

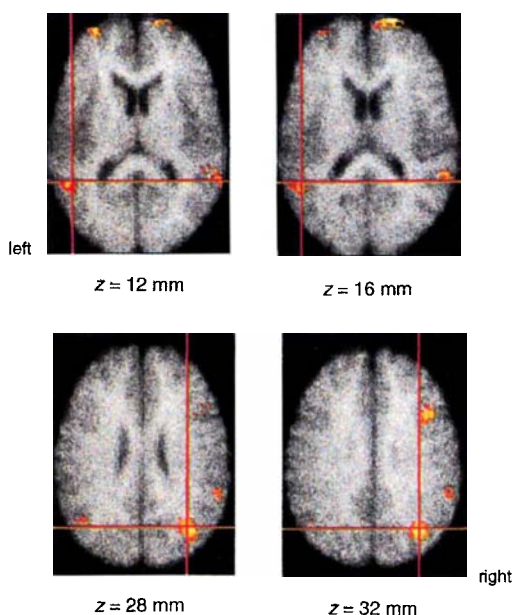
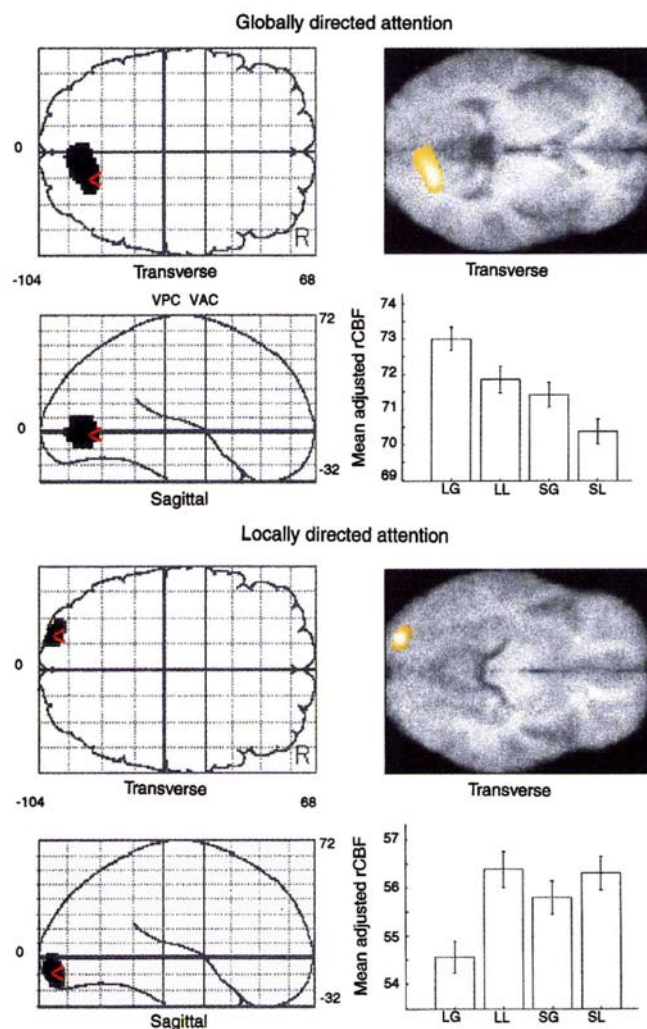


FIG. 3 Relative rCBF increases (for the 6 subjects) seen in experiment 2. Areas of significant relative rCBF increase ($P < 0.01$) are shown superimposed upon transverse sections of the group mean MR-image that had been spatially normalized into the same anatomical space¹⁵. The red cross-hair indicates the local maxima within the areas of activation in the posterior part of the left superior temporal gyrus (upper row) and in the temporal-parietal-occipital junction (lower row). z, distance above the intercommissural (AC-PC) line in mm. The exact coordinates of the local maxima and their z statistics are given in Table 1.

METHODS. Six normal-sighted male right-handed volunteers were studied using identical equipment, data acquisition and processing, as in experiment 1 (Fig. 2). SPM95 (ref. 19) was used to assess significant covariation of relative rCBF with the number of target switches from either the global to the local level (or vice versa). The resulting SPM $\{z\}$ was thresholded at $P < 0.01$ (uncorrected) as the experiment was specifically concerned with the temporal-parietal areas. Other activations are not reported.

Variation in the number of target switches significantly co-varied, as predicted, with temporal-parietal (but not with pre-striate) activation. The number of successive stimuli for which subjects had to sustain attention to either the global or the local level was significantly correlated with blood-flow increases in the temporal-parietal cortex on the right ($P < 0.001$, Fig. 3, Table 1) and on the left ($P < 0.01$, Fig. 3, Table 1).

Our findings indicate that globally directed attention involves the right hemisphere, and locally directed attention the left hemisphere, confirming the indications of previous studies^{2,5}. A striking feature of our findings is that the hemispheric processing differences are apparent in the early stages of visual processing in pre-striate cortex, even though the visual stimuli were invariant across conditions. Our observations thus extend previous reports on selective attention⁶⁻⁸ in that they demonstrate a 'top-down' modulation of the processing of identical input in early visual areas. The areas activated differentially in the lingual gyrus (global processing) and the inferior occipital cortex (local processing) encroach onto the cuneus but do not include the striate cortex (area V1). According to human anatomical and imaging evidence, the focus in the left inferior occipital cortex falls between V1/V2 and V2/V3 boundaries, whereas the focus in the right lingual gyrus falls anterior to the V2/V3 boundaries⁹⁻¹¹. In the second experiment, the activation of non-homologous regions in the right temporal-parietal-occipital junction (BA 39/19) and in the left posterior aspect of the superior temporal gyrus (BA 22/39) during the divided attention task is of particular interest: it is predicted by the locus of lesions in patients with impaired global and local processing abilities^{2,5}. Temporal-parietal regions seem to exert attentional control over global and local processing.

Our observations imply that neuronal activity in early visual areas does not only represent the sensory attributes of a retinal image^{8,12}. The independent effects of stimulus size and global processing on the neuronal activation in the right lingual gyrus contrasts with the interaction of stimulus size and local processing in the left inferior occipital cortex. It is evident that the activations are not solely determined by stimulus size (or correlated differences in spatial frequencies), which was controlled across conditions. One explanation for the observed interaction is that for small hierarchical stimuli, even the global form has a strong local aspect by virtue of retinal size. The differential activations observed may thus reflect attentional modulation of the respective retinotopic fields within V2 and V3 during global and local processing. For any specific complex visual stimulus, global processing necessarily involves a larger peripheral visual field and local processing involves the central visual field.

Reports of impaired global processing in patients with right

TABLE 1 Brain activity associated with global and local processing during attentional tasks

Region	Side	Coordinates			Z-score
		x	y	z	
Experiment 1 (A + C) – (B + D): globally directed attention					
Lingual gyrus (BA 18)	R	16	–74	0	5.0
Experiment 1 (B + D) – (A + C): locally directed attention					
Inferior occipital gyrus (BA 18)	L	–22	–96	–8	5.3
Experiment 2: divided attention					
Temporal–parietal–occipital junction (BA 39/19)	R	32	–72	32	3.5
Superior temporal gyrus (BA 22/39)	L	–48	–54	16	2.9

In experiment 1, coordinates (in standard stereotactic space¹⁵) refer to maximally activated foci as indicated by the highest Z-score within an area of activation associated with globally or locally directed attention. In experiment 2, coordinates refer to maximally activated foci associated with the number of successive stimuli to which subjects had to sustain attention to either global or local levels during a divided attention task (for details see Fig. 1). x, distance (mm) to right (+) or left (-) of the midsagittal line; y, distance anterior (+) or posterior (-) to vertical plane through the anterior commissure; z, distance above (+) or below (-) the intercommissural (AC-PC) line. For each anatomical location, an estimate of the Brodmann area (BA) is given in parentheses¹⁵. R, right; L, left. Coordinates of the border of V1/V2 are -10, -98, -4, and of V2/V3 are -20, 78, -4 (ref. 10).

temporal-parietal lesions (but without visual-field deficits) and impaired local processing with similar left temporal-parietal lesions support the concept of hemispheric asymmetry and suggest that the temporal-parietal regions are critical for local and global processing^{2,5}. However, the interpretation of lesion studies is limited by compensatory mechanisms and both local and global processing can be preserved after large unilateral brain lesions¹³. The absence of a differential temporal-parietal activation during the global and local tasks in our first experiment with normal subjects is most probably a result of presenting stimuli in blocks. In situations where the subject does not know in advance the level at which the relevant stimulus will occur, the temporal-parietal cortices may mediate the voluntary distribution and/or maintenance of selective attention^{4,14}.

In our second experiment, we confirmed this prediction by demonstrating temporal-parietal activations in a divided attention task. We show that the modulatory influence of temporal-parietal activity during global and local processing has its predominant effect in pre-striate cortex, at an early stage of visual processing. Damage to temporal-parietal cortex can accordingly impair either global or local processing because of the attentional control that these areas exert over the computations performed in pre-striate cortex. □

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- Navon, D. *Cogn. Psychol.* **9**, 353-383 (1977).
- Robertson, L. C., Lamb, M. R. & Knight, R. T. *J. Neurosci.* **8**, 3757-3769 (1988).
- Marshall, J. C. & Halligan, P. W. *Nature* **373**, 521-523 (1995).
- Hellige, J. B. *Hemispheric Asymmetry: What's Right and What's Left* (Harvard Univ. Press, Cambridge, MA, 1993).
- Robertson, L. C. & Lamb, M. R. *Cogn. Psychol.* **23**, 299-330 (1991).
- Heinze, H. J. et al. *Nature* **372**, 543-546 (1994).
- Corbetta, M., Miezin, F. M., Dobmeyer, S., Shulman, G. L. & Petersen, S. E. *Science* **248**, 1556-1559 (1990).
- Motter, B. C. *J. Neurophysiol.* **70**, 909-919 (1993).
- Clarke, S. *Eur. J. Neurosci.* **6**, 725-736 (1994).
- Shipp, S., Watson, J. D. G., Frackowiak, R. S. J. & Zeki, S. *Neuroimage* **2**, 125-132 (1995).
- Sereno, M. I. et al. *Science* **268**, 889-893 (1995).
- Moran, J. & Desimone, R. *Science* **229**, 782-784 (1985).
- Polster, M. R. & Rapcsak, S. Z. *Cortex* **30**, 487-497 (1994).

- Rafal, R. & Robertson, L. C. in *The Cognitive Neurosciences* (ed. Gazzaniga, M. S.) 625-647 (MIT Press, London, 1995).
- Talairach, J. & Tournoux, P. *Coplanar Stereotaxic Atlas of the Human Brain* (Thieme, Stuttgart, 1988).
- Tipper, S. P., Weaver, B. & Houghton, G. Q. *J. Exp. Psychol.* **A 47**, 809-840 (1994).
- Silbersweig, D. A. et al. *J. Cereb. Blood Flow Metab.* **13**, 617-629 (1993).
- Friston, K. J. et al. *Hum. Brain Map.* **2**, 1-25 (1995).
- Friston, K. J. et al. *Hum. Brain Map.* **2**, 189-210 (1995).

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CORRESPONDENCE and requests for materials should be addressed to R.J.D. (e-mail: r.dolan@fil.ion.ucl.ac.uk).

Reward expectancy in primate prefrontal neurons

Masataka Watanabe

Department of Psychology, Tokyo Metropolitan Institute for Neuroscience, Musashidai 2-6, Fuchu, Tokyo 183, Japan

THE prefrontal cortex is important in the organization of goal-directed behaviour¹⁻³. When animals are trained to work for a particular goal or reward⁴⁻⁷, reward 'expectancy' is processed by prefrontal neurons. Recent studies of the prefrontal cortex have concentrated on the role of working memory in the control of behaviour⁸⁻¹⁰. In spatial delayed-response tasks, neurons in the prefrontal cortex show activity changes during the delay period between presentation of the cue and the reward¹¹⁻¹⁵, with some of the neurons being spatially specific (that is, responses vary with the cue position)¹³⁻¹⁵. Here I report that the delay activity in prefrontal neurons is dependent also on the particular reward received for the behavioural response, and to the way the reward is given. It seems that the prefrontal cortex may monitor the outcome of goal-directed behaviour.

When a monkey was presented with a non-preferred food item (lettuce) after making a correct response to the position where a preferred food item (banana) had been shown in a delayed-response task, the animal displayed surprise and anger⁴. It must have remembered and expected the specific reward (banana), as well as remembering where it was presented. The animal seems to acquire through learning the expectancy that a particular behaviour leads to a particular outcome⁵.

I trained two monkeys in a delayed-response task with several kinds of food and liquid rewards to examine reward-specific anticipatory neuronal activity. Three different types of tasks were used: (1) visible food, (2) stimulus associated with food, and (3) stimulus associated with liquid (Fig. 1a), to investigate whether there were differences in reward-specific anticipatory activity depending on whether the reward was shown during the cue period. Neuronal activity was recorded from the dorsolateral prefrontal cortex (Fig. 1b).

Of 124 prefrontal 'delay neurons' in which I recorded delay-related activity changes in at least one type of task, 82 showed spatial specificity. I recorded from 42 prefrontal neurons during tests using more than 3 different kinds of reward, for at least one type of task. Half of the neurons (21) showed different activity changes with different rewards. Such reward-dependent activity was observed in delay neurons both without (5/11) and with (16/31) spatial specificity (Figs 2 and 3, respectively).

Reward-dependent activity changes during the delay were observed not only in the 'visible food' task (15 of 25 neurons examined) but also in the 'stimulus associated with food or liquid' task (13 of 31 neurons examined). The majority of reward-dependent delay neurons (17 of 21) showed more activity changes when the monkey was given a preferred than a non-preferred reward. In those delay neurons ($n = 11$) that showed reward dependency in both the 'visible food' and 'stimulus associated with food' tasks, preference for the different kinds of food rewards was the same between these tasks. As in ref. 4, monkeys expected a specific reward during the delay period. The observed reward-dependent anticipatory activity is probably related to this expectancy, which may consist of retrieving, retaining and/or anticipating the motivational value and visual, gustatory and/or olfactory images of the specific reward.

In reward-dependent delay neurons that showed spatial specificity, there was no tendency for the magnitude of delay-related activity changes to be related to the discrimination in neuronal activity between right and left stimulus locations. Thus, spatial and reward information are probably processed independently in those neurons.

Reward-related delay activity was observed not only within each task but also among the different types of delayed response tasks. Of the 42 delay neurons, 22 were examined on at least two different kinds of rewards in all three task types. The majority (17 of 22, 13 of them showing within-task reward dependency as well) showed task-dependent differential activity. Six neurons showed a different pattern or magnitude of activity changes depending on whether the reward was visible or signalled by a stimulus. The neuron represented in Fig. 4a showed delay-related activity changes with spatial specificity in the 'visible food' task but not in 'stimulus associated with food' task, although the food was not visible during the delay in either task.

Eight neurons showed a different pattern or magnitude of activity changes between food- and liquid-reward tasks irrespective of whether the reward was visible or not (for example Fig. 4b), and another three neurons gave distinct responses for all three different types of tasks. Thus, expectancy of the specific reward

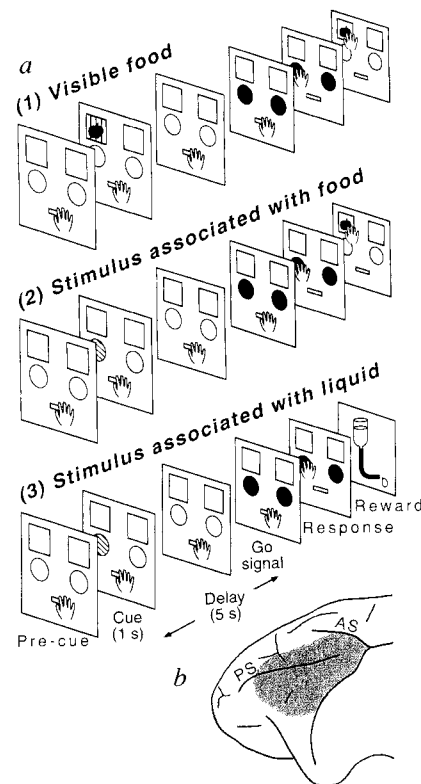


FIG. 1 a Sequences of events in the three different types of delayed-response tasks. b, Area in the prefrontal cortex (shaded area) from which neurons were recorded. PS, principal sulcus; AS, arcuate sulcus.

METHODS. The animal (*Macaca fuscata*) was seated on a primate chair facing a panel which contained right and left rectangular windows, circular keys and a hold lever below them. Each window contained two screens; one opaque and one transparent with thin vertical lines. The animal first depressed the lever for 6–8 s, and a food reward was presented on the right or left window behind the transparent screen (1, visible food), or a red light was presented on the right or left key (2 and 3, stimulus associated with food or liquid), as a cue for 1 s. There was then a delay period of 5 s, which was followed by a 'go' signal of white lights on both keys and the animal was required to respond to the cued side. Correct responses were rewarded with the food or liquid which had been presented during the cue period (1, Visible food), or which had been prepared (2 and 3, Stimulus associated with food or liquid). During the recording, the type of task was changed, without warning, after blocks of about 50 trials. A piece (~0.5 g) of raisin, sweet potato, cabbage or apple was used as a food reward, and a drop (~0.4 ml) of water, refreshing (sweet isotonic) beverage, orange juice or grape juice, delivered through a spout at the animal's mouth, was used as a liquid reward. The same kind of reward was usually used for about 50 trials. Procedures for surgery, recording and data analysis were as described^{21,22}.

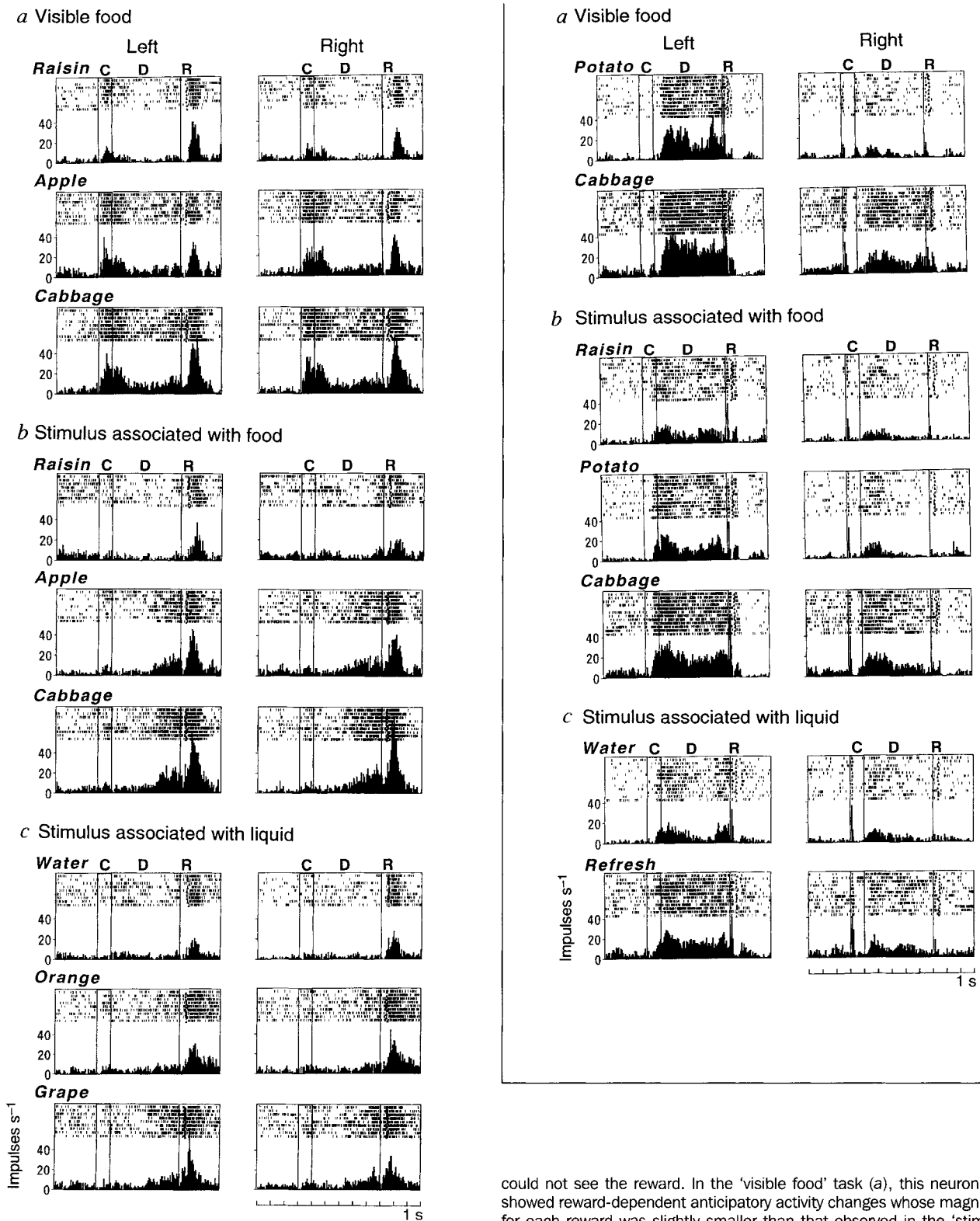
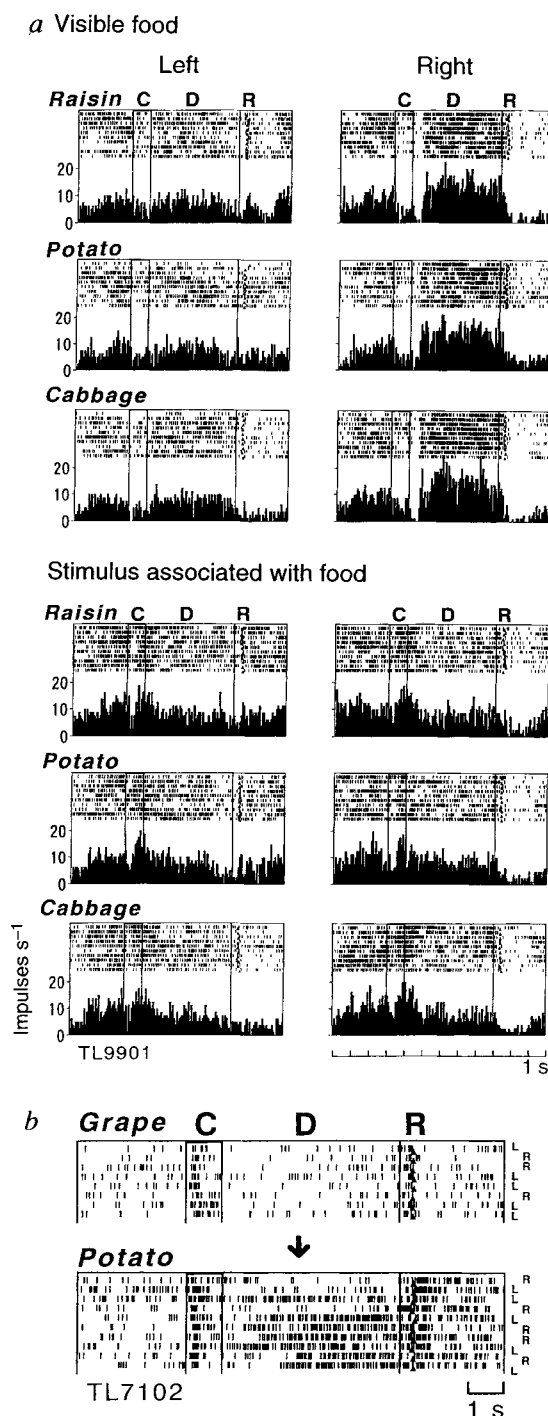


FIG. 2 An example of a reward-dependent prefrontal delay neuron displaying no spatial specificity in the three task types. *a*, Visible food; *b*, Stimulus associated with food; *c*, Stimulus associated with liquid. In the 'stimulus associated with food' task (*b*), this neuron showed an increase in anticipatory activity changes on cabbage- and apple- but not on raisin-rewarded trials. The increase with cabbage and apple rewards was not significantly different. The animal could retrieve and anticipate the specific reward as the same reward was used in a block of about 50 trials. On the 'stimulus associated with liquid' task (*c*), a similar pattern of reward-dependent differential delay activity was observed even though the animal

could not see the reward. In the 'visible food' task (*a*), this neuron also showed reward-dependent anticipatory activity changes whose magnitude for each reward was slightly smaller than that observed in the 'stimulus associated with food' task and showed, in addition, reward-dependent cue-related differential activity. The magnitude of activity changes observed after the delivery of each reward was in the same order as during the delay period. Neuronal activity is shown in raster and histogram displays. For each display, the second and third vertical lines indicate cue onset and offset, and the fourth vertical line indicates the end of the delay period. Each row indicates one trial, and small upward triangles in the raster indicate the time of the key-pressing responses. Only data from correct trials are shown. The data from the first trial after changing the type of task and/or the kind of reward are omitted from the analysis. Left- and right-side displays are shown separately. The reward used is stated. C, cue; D, delay. R, response.

◀ FIG. 3 An example of a reward-dependent delay neuron displaying spatial specificity in three task types. *a*, Visible food; *b*, Stimulus associated with food; *c*, Stimulus associated with liquid. This neuron showed differential anticipatory activity changes with the different rewards in the left-side trials. The order of food preference was cabbage, potato and raisin in the 'stimulus associated with food' task (*b*), and the refreshing beverage was preferred to water in the 'stimulus associated with liquid' task (*c*). The magnitude of activity changes during the delay period was not significantly different between the 'visible food' (*a*) and 'stimulus associated with food' (*b*) tasks with the potato and cabbage rewards, although activity changes started much earlier on the latter task. Although there was no significant difference in activity at the start of each trial between different reward conditions, this neuron showed a slightly higher rate of activity changes

during the pre-cue period with cabbage and refreshing beverage trials than other reward trials. Such reward-specific pre-cue anticipatory activity was observed in roughly half (11/21) of reward-dependent delay neurons. For each reward-dependent delay neuron with spatial specificity, the 'discrimination index' of $[(A - B)/(A + B)]$ was calculated for each reward condition, where A and B indicate the mean discharge rate during the delay period for right and left trials, and a higher index indicates better discrimination between right and left. For this neuron, discrimination index was 0.38, 0.46 and 0.31 for the raisin, potato and cabbage rewards, respectively, in the 'stimulus associated with food' task. For the delay neurons that had both reward dependency and spatial specificity, there was no significant tendency for the magnitude of reward-dependent activity changes to be related to the magnitude of the index. Abbreviations as Fig. 2.



◀ FIG. 4 *a*, An example of a delay neuron that showed a different pattern of delay-related activity changes in different types of delayed response tasks. *b*, An example of a delay neuron that showed a different magnitude of delay-related activity changes between food and liquid reward tasks, and whose activity was examined as the reward was changed. The delay neuron in *a* showed spatial specificity without showing food-reward dependency in the 'visible food' task (top panels) while showing neither spatial specificity nor reward dependency on the 'stimulus associated with food' task (lower panels) (and on the 'stimulus associated with liquid' task as well; data not shown). The neuron in *b* showed delay-related activity changes without spatial specificity on food-reward trials (irrespective of whether the reward was visible or not), but not on liquid-reward trials. This neuron was examined, in a non-visible reward situation, before and after the reward change from grape juice (upper raster) to potato (lower raster). On the first trial after the reward change, delay-related activity was quite similar to that observed on former trials as the animal did not know the reward change. As was always the case for reward-related delay neurons, this neuron showed an activation after delivery of the unexpected reward. The activity on the second trial was still similar to that on the first trial, maybe because the animal did not learn that the reward had changed. In the third trial, this neuron showed an activation during the delay period, and the activation after the reward delivery decreased. The transition of neuronal activity after the reward change occurred usually within 2 or 3 trials, although there was sometimes fluctuation during the transition process as was the case for this neuron. L and R to the side of the rasters indicate left and right trial, respectively. Other abbreviations as Fig. 2.

may be attained by the ensemble of activities of reward- and task-dependent differential delay neurons.

During the recording I sometimes delivered an unexpected reward in the 'visible food' task (as in ref. 4), which upset the behaviour of the monkey, and triggered neuronal activation that continued for a minute or so, whereas reward-related delay activity was absent in those trials. It was possible, however, in the 'stimulus associated with food or liquid' tasks to follow the transition in the activity of reward-related delay neurons during the change of the reward and of the animal's expectancy (Fig. 4b).

Anticipatory changes in neuronal activity in relation to task events such as cue or reward presentation have been reported in the primate prefrontal cortex^{16,17} and the basal ganglia¹⁸⁻²⁰. I have previously demonstrated differential delay activity in the monkey prefrontal cortex depending on whether or not a reward was expected^{21,22}. Prefrontal neurons were also shown to code the consequence of the response (reinforcement or error)^{16,23}. Here, examination of dorsolateral prefrontal neurons using several kinds of rewards has indicated for the first time that they are involved in the 'expectancy' of the specific reward, in other words, goal for the behavioural response. The existence of task-dependent differential neuronal responses also indicates that the dorsolateral prefrontal cortex is important in monitoring the context of the situation in relation to how and what reward is given. Patients with prefrontal lesions are poor at assessing how actions relate to goals²⁴; deficits of this kind may be caused by the loss of such prefrontal neurons. The fact that prefrontal neurons are involved

in retaining the spatial working memory and in reward expectancy indicates that the prefrontal cortex is processing different kinds of information in parallel. □

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1. Luria, A. R. *Higher Cortical Functions in Man* 2nd edn (Basic Books, New York, 1980).
2. Shallice, T. *From Neuropsychology to Mental Structure* (Cambridge Univ. Press, New York, 1988).
3. Fuster, J. M. *The Prefrontal Cortex: Anatomy, Physiology, and Neuropsychology of the Frontal Lobe* 2nd edn (Raven, New York, 1989).
4. Tinklepaugh, O. L. *J. comp. Psychol.* **8**, 197–236 (1928).
5. Tolman, E. C. *Purposive Behavior in Animals and Men* (Century, New York, 1932).
6. Peterson, G. B. in *Animal Cognition* (eds Roitblat, H. L., Bever, T. G. & Terrace, H. S.) 135–148 (Lawrence Erlbaum Associates, Hillsdale, 1984).
7. Chatlosh, D. L. & Wasserman, E. A. in *Learning and Memory* (eds Germezeno, I. & Wasserman, E. A.) 61–79 (Lawrence Erlbaum Associates, Hillsdale, 1992).
8. Goldmann-Rakic, P. S. in *Handbook of Physiology Vol. 5 The Nervous System, Higher Functions of the Brain Part 1* (ed. Plum, F.) 373–417 (Am. Physiol. Soc., Bethesda, 1987).
9. Petrides, M., Alivisatos, B., Evans, A. C. & Meyer, E. *Proc. natn. Acad. Sci. U.S.A.* **90**, 873–877 (1993).
10. McCarthy, G. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 8690–8694 (1994).
11. Kubota, K. & Niki, H. *J. Neurophysiol.* **34**, 337–347 (1971).
12. Fuster, J. M. *J. Neurophysiol.* **36**, 61–78 (1973).
13. Niki, H. *Brain Res.* **70**, 346–349 (1974).
14. Niki, H. & Watanabe, M. *Brain Res.* **105**, 79–88 (1976).
15. Funahashi, S., Bruce, C. J. & Goldman-Rakic, P. S. *J. Neurophysiol.* **61**, 331–349 (1989).
16. Niki, H. & Watanabe, M. *Brain Res.* **171**, 213–224 (1979).
17. Sakagami, M. & Niki, H. *Expl. Brain Res.* **97**, 423–436 (1994).
18. Hikosaka, O., Sakamoto, M. & Usui, S. *J. Neurophysiol.* **61**, 814–832 (1989).
19. Apicella, P., Scarnati, E., Ljungberg, T. & Schultz, W. *J. Neurophysiol.* **68**, 945–960 (1992).
20. Schultz, W., Apicella, P., Scarnati, E. & Ljungberg, T. *J. Neurosci.* **12**, 4595–4610 (1992).
21. Watanabe, M. *Expl. Brain Res.* **80**, 296–309 (1990).
22. Watanabe, M. *Expl. Brain Res.* **89**, 233–247 (1992).
23. Watanabe, M. *Neurosci. Lett.* **101**, 113–117 (1989).
24. Singu, A. et al. *Cortex* **31**, 301–316 (1995).

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CORRESPONDENCE and requests for information should be addressed to the author (e-mail: masataka@trn.ac.jp).

Visual projection map specified by topographic expression of transcription factors in the retina

Junichi Yuasa, Shinji Hirano, Masahito Yamagata & Masaharu Noda

Division of Molecular Neurobiology, National Institute for Basic Biology, and Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Myodaiji-cho, Okazaki 444, Japan

TOPOGRAPHICAL maps of neuronal connectivity occur in various brain regions¹. In the visual system of birds, retinal ganglion-cell axons from the anterior retina connect to a posterior part of the optic tectum, and posterior retinal axons connect to the anterior part, thereby establishing a point-to-point projection map^{2,3}. The chemoaffinity theory⁴ predicts that the orderly retinotectal projection is generated by a topographical arrangement of molecules. We report here that we have found several genes topographically expressed along the nasotemporal (anterior–posterior) axis in the embryonic chicken retina. Among these, two transcriptional regulators, belonging to the winged-helix family⁵ are expressed in a mutually exclusive manner in either the nasal or temporal part of the retina. Misexpression of each factor causes misprojection on the tectum along the rostrocaudal axis, showing that topographical expression of these transcription factors controls formation of the retinotectal map.

The winged-helix (WH) domain is a DNA-binding motif found in the family of transcription factors containing vertebrate HNF-3 and *Drosophila* forkhead⁵. The nasal-specific transcript isolated

here, N-62-5 (Fig. 1a), is homologous to rat brain-factor-1 (BF-1) (overall identity, 91%; WH domain, 99%)⁶ and is identical to the *c-qin* protein⁷, the cellular counterpart of the avian retroviral oncogene *v-qin*⁸. Our temporal-specific molecule T-14-6 (Fig. 1a) is 67% identical to mouse BF-2 (ref. 9) (Fig. 1b), with a highly conserved (99%) WH domain. We tentatively refer to it as a chicken homologue of BF-2 (CBF-2). For consistency, we will refer to N-62-5 as chicken BF-1 (CBF-1). As shown in Fig. 1c, CBF-1 and CBF-2 are also classified by the position of an additional conserved motif, named region II⁵. Mutually exclusive expression of two distinct WH-domain-containing transcriptional regulators in either the nasal or temporal part of the retina suggests that they may determine regional specificity in the retina.

CBF-1 and CBF-2 transcripts are present by developmental stage 9 (ref. 10). They can be detected topographically in regions including primordial retina and neuroepithelium by embryonic day 2 (E2; stages 10–11; Fig. 2a, d), preceding expression of other reported topographically distributed molecules in the retina. At E3 (stages 16–19), CBF-1 was clearly expressed in the nasal retina and pigment epithelium, as well as in the telencephalon (Fig. 2b, c). During the same stages, CBF-2 was expressed in the temporal retina and associated pigment epithelium as well as in part of the diencephalon (Fig. 2e, f). The expression patterns are reminiscent of those in mice⁹. At E7, both transcripts are deposited on the vitreous side of the retina (not shown), indicating expression in retinal ganglion cells.

Northern-blot analysis showed their expression in the retina to decline from E4, almost disappearing by E10 (not shown). Retinal axons begin to enter the tectum at E6 and develop synaptic connections from E12 (ref. 11), thus expression of these factors ceases during the onset of retinotectal connection. However, as nasotemporal specificity of the retina is determined at stage 11 (E2)¹², we suggest that CBF-1 and CBF-2 determine the naso-temporal axis of the retina, and consequently specify the topographical projection of the retinal ganglion-cell axons to the tectum by controlling expression of their target genes.

We expressed CBF-1 or CBF-2 complementary DNA in the retina with a replication-competent subgroup-B avian retroviral vector^{13–15}. Recombinant virus was injected into the prosencephalon at stage 9–11 (E2). Infection and expression were monitored by immunostaining for the retroviral *gag* protein and by *in situ* hybridization with probes for CBF-1 or CBF-2. Infection and expression were observed (70–90% of cells) in the whole retina by E4 (Fig. 3a, b, d, e). By E18, infected cells spread selectively throughout the entire retinal ganglion-cell layer (Fig. 3c). As virus injection was restricted to the presumptive forebrain and eye, infected cells were scarcely found in the tectum (not shown).

We analysed the retinotopic tectal map at E19–20 by labelling the nasal and temporal retina with DiI^{16,17}. By this time the retinotectal projection is normally established (see Fig. 4 legend). As expected, in the embryos infected with the CBF-1 recombinant virus, temporal axons frequently passed their appropriate terminal zones of the rostral tectum, and extended caudally (6 of 20 embryos). Branched collaterals and, in some cases, terminal arborization and synaptic varicosities, were observed in the caudal tectum (2 of 20 embryos; Fig. 4a). By contrast, nasal axons behaved normally, and never arborized onto the rostral side (22 embryos). In control embryos injected with an alkaline phosphatase cDNA-bearing virus (*n* = 22) or CBF-2 virus (*n* = 21), no aberrant projections of temporal axons were observed. We also studied retinal ganglion cells by retrograde labelling using DiI crystals placed across the mediolateral axis on the caudal tectum at E18 (Fig. 4B). In control embryos, all ganglion cells labelled from the caudal tectum selectively populated the nasal retina, and no ganglion cells were found labelled in the temporal retina (Fig. 4Bb, d). In the CBF-1 virus-infected retina, many labelled ganglion cells were detected in the temporal retina (Fig. 4Be, g, h), indicating that a large number of temporal ganglion-cell axons project to the caudal tectum (22 of 24 embryos).

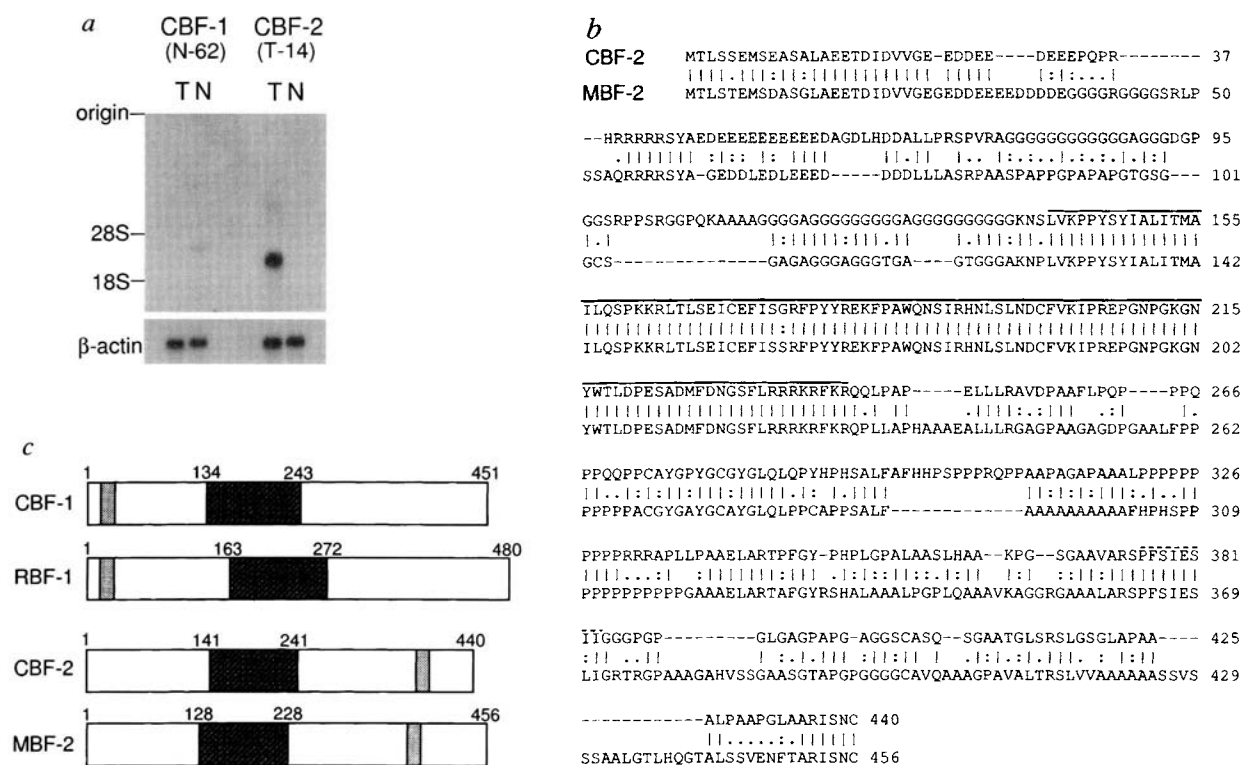


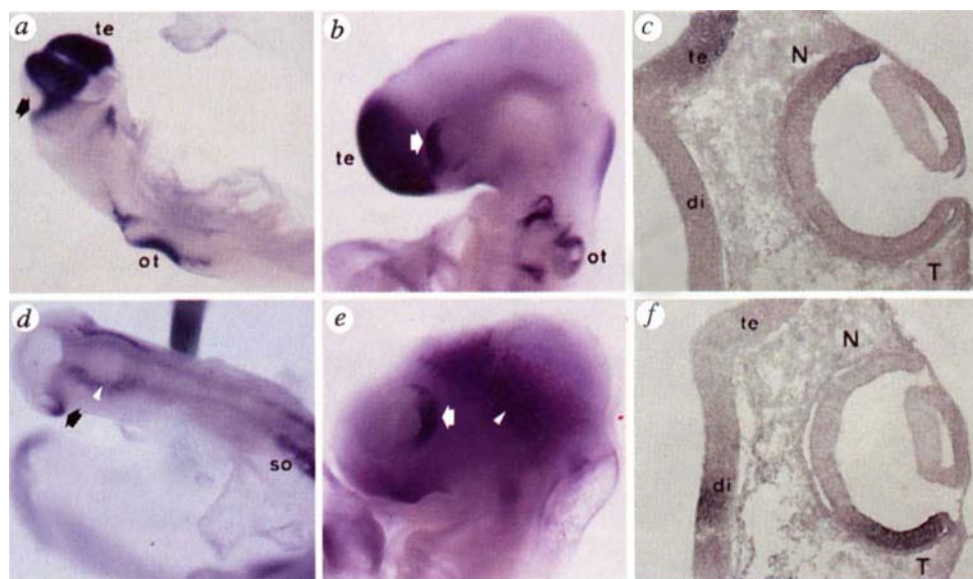
FIG. 1 Isolation of CBF-1 and CBF-2 by subtractive hybridization²⁸. **a**, CBF-1 and CBF-2 mRNA expression in the retina. Poly(A)⁺ RNA was isolated from the nasal (N) or temporal (T) third of E8 retina. RNA blot hybridization analysis (3 μ g per lane) was done by probing with an N-62 or T-14 insert. Transcripts were seen at 3.1 kilobases (kb) for CBF-1, and 2.5 kb (major) and 6.3 kb (minor) for CBF-2. Blots were rehybridized with chicken β -actin cDNA to verify the amount of mRNA loaded in each lane. **b**, Amino-acid sequence comparison (single-letter code) of CBF-2 with mouse BF-2. Gaps (—) were inserted to maximize homology. Exact matches are indicated by bars and conservative substitutions by colons (comparison value ≥ 0.50) and dots (≥ 0.10)²⁹. The WH domain (solid overline) and region II (dashed

overline) are marked. **c**, Schematic diagrams of BF-1 and BF-2. Hatched boxes, WH domain; shaded boxes, region II; residue numbers are shown above. RBF-1, rat BF-1; MBF-2, mouse BF-2.

METHODS. From the subtracted libraries, N-62 and T-14 were first identified as major clones that are specifically expressed in the nasal and temporal retina, respectively. Another cDNA library of chick retina was screened to obtain the full-length clones, N-62-5 (CBF-1) and T-14-6 (CBF-2). See Supplementary Information for details. We also isolated three independent clones, showing temporal retina-specific expression (data not shown). GenBank accession numbers: U47275 (CBF-1) and U47276 (CBF-2).

FIG. 2 CBF-1 and CBF-2 mRNA expression in chick embryos. Whole-mount *in situ* hybridization of stage 11 (**a**, **d**) and stage 16–17 (**b**, **e**) embryos, and *in situ* hybridization on horizontal sections through the head portion at E3 (stage 19; **c**, **f**) probed with either CBF-1 (**a**, **b**, **c**) or CBF-2 (**d**, **e**, **f**). By stage 11, CBF-1 was expressed in the anterior prosencephalon and the anterior region of the primordial optic vesicle (**a**, arrow) as well as in the otic placode, and CBF-2 was expressed in the posterior optic vesicle (**d**, black arrow) and in somites. In stage 16–17 embryos, CBF-1 was clearly expressed in the nasal eye (**b**), and CBF-2 in the temporal eye (**e**, white arrows). The arrowheads in **d** and **e** show signals in mesenchyme, verified by transverse sectioning. At E3 (stage 19), CBF-1 was expressed in the nasal (N) retina and telencephalon (**c**), and CBF-2 was expressed in the temporal (T) retina and diencephalon (**f**). Control sense probes gave no specific hybridization signals (not shown). te, Telencephalon; ot, otic vesicle; so, somites; di, diencephalon.

METHODS. The hybridization was as described³⁰. Digoxigenin-labelled riboprobes were synthesized using full-length cDNAs of CBF-1 and CBF-2



in pBluescript as templates, and hybridized to paraformaldehyde-fixed embryos or cryosections. Digoxigenin was detected with alkaline phosphatase-labelled anti-digoxigenin antibodies, followed by staining with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate.

FIG. 3 Retroviral infection of chick embryos. Infection and expression rate in the retina were examined in horizontal sections through the head of E4 embryos (48 h after injection). *a*, DAPI (4', 6-diamidino-2-phenylindole) staining of the CBF-1/RCAS(B)-infected embryo to visualize nuclei. *b*, Expression of the viral *gag* protein in CBF-1/RCAS(B)-infected embryos detected immunohistochemically. The field is as *a*. *c*, *gag* protein expression in a retinal section of the E19 embryo infected with CBF-1/RCAS(B). The ganglion-cell layer showed strong expression. *d*, *In situ* hybridization of CBF-1/RCAS(B)-infected retina, probed with CBF-1. *e*, *In situ* hybridization of CBF-2/RCAS(B)-infected retina, probed with CBF-2. Inset is a higher magnification view of the area where CBF-1 (*d*) or CBF-2 (*e*) is expressed ectopically (arrowheads). In, lens; nr, retina; pe, pigment epithelium; di, diencephalon; inl, inner nuclear layer; gcl, ganglion-cell layer. Scale bar, 100 μ m except for *c* (25 μ m) and insets in *d*, *e* (50 μ m).

METHODS. The cDNAs containing the coding region of CBF-1, CBF-2 and human placental alkaline phosphatase were cloned into the *Cla*I site of RCASBP(B) plasmid vector^{13,15} to yield CBF-1/RCAS(B), CBF-2/RCAS(B) and AP/RCAS(B), respectively. Concentrated virus stocks ($\sim 10^8$ infectious units per ml) were prepared as described¹⁴. Virus solution, supplemented with 80 μ g per ml polybrene (Sigma), was injected into the prosencephalic ventricle of C/O (chicken strain not resistant to any retrovirus subgroup) chick embryos (Nisseiken and Biken) *in ovo*^{14,15}, and infected embryos were fixed with 4% paraformaldehyde at the described stages. The infected retina showed normal cytoarchitecture, ganglion cells expressed cytochemical markers such as neurofilaments, and the tectum showed apparently normal polarity in terms of gross morphology and laminar architecture. Sections were immunostained with the 3C2 monoclonal antibody against the viral *gag* protein (Developmental Studies Hybridoma Bank), and visualized with FITC-conjugated anti-mouse immunoglobulin antibodies (Jackson).

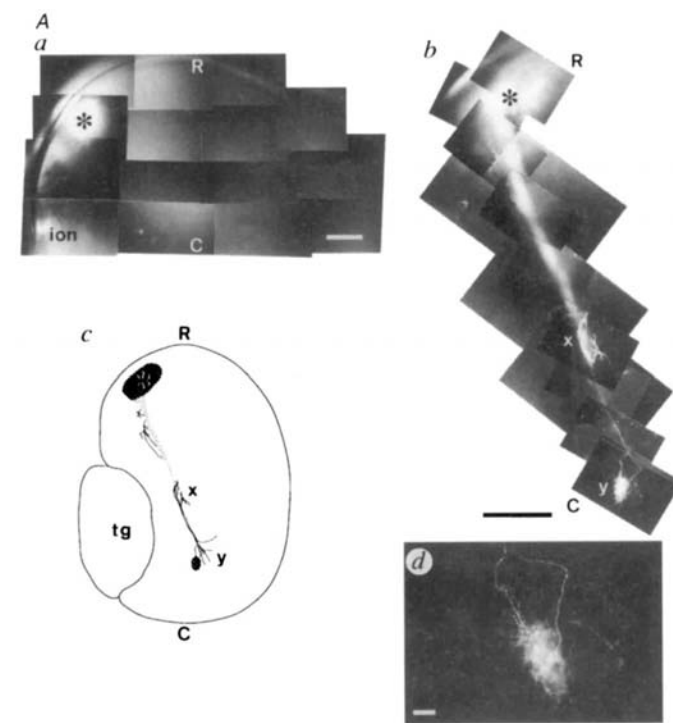
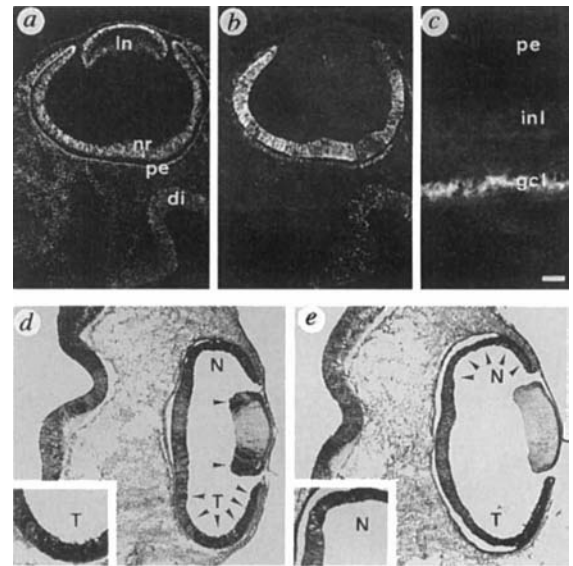
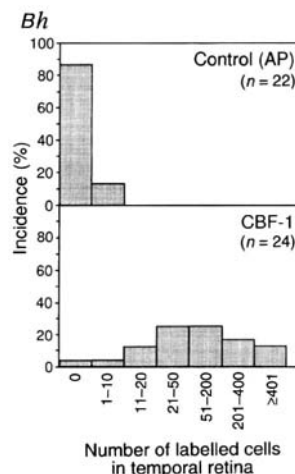
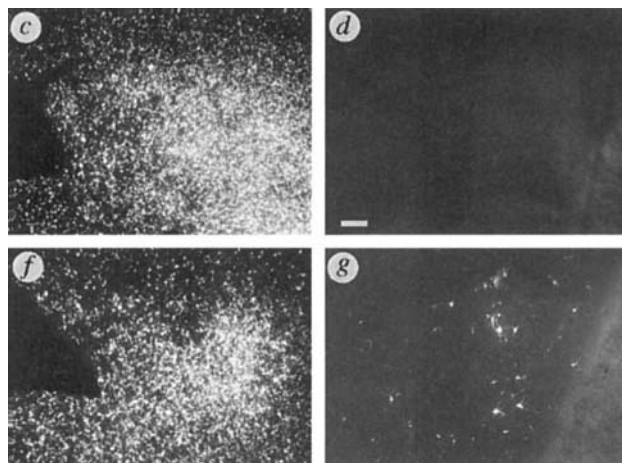
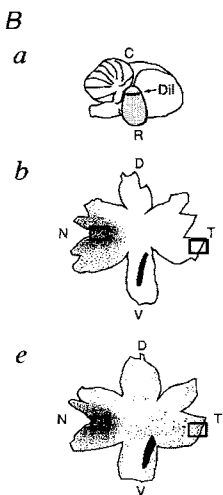


FIG. 4 Ectopic expression of CBF-1 and CBF-2 results in alterations of retinotectal projections. *A*, Projections of the temporal retinal axons. *a*, Control AP/RCAS(B) virus. *b*, CBF-1/RCAS(B)-infected embryo. Some temporal axons extended caudally over the appropriate terminal zone (*) and developed ectopic arborization (x) and (y). *c*, Schematic drawing of *b*. *d*, Higher magnification of region (y) in *b*. *B*, Retrogradely labelled retinal ganglion cells. Dil crystals were placed as shown in *a*. Schematic representation of labelled retinal ganglion cells in control (*b*) and CBF-1/RCAS(B)-infected (*e*) embryos. Real images of the nasal and temporal areas boxed in *b* are shown in *c* and *d*, and those in *e* are shown in *f* and *g*. Temporal retinæ in CBF-1/RCAS(B)-infected embryos contained a large number of labelled ganglion cells (*g*). The data are shown as histograms in *h*. *C*, Projection of nasal axons. *a*, Control AP/RCAS(B)-infected embryo. *b*, CBF-2/RCAS(B)-infected embryo. The ectopic arborization and deflected axonal trajectory are marked by a white arrow and arrowheads, respectively. *c*, Higher magnification of the ectopic arborization in *b*. ion, Isthmo-optic nucleus, retrogradely labelled from retina; tg, tegmentum; dc, diencephalic visual centre; R, rostral; C, caudal. Scale bars, 500 μ m in *Aa*, *b*, *Ca* and *b*; 100 μ m in *Bc*, *d*, *f*, *g*; 50 μ m in *Ad* and *Cc*. Methods are reported in Supplementary Information. Some temporal retinal axons overshoot and branch in the caudal tectum before E13, but such misprojections are absent by E15 in normal development¹⁷.



In embryos infected with the CBF-2 recombinant virus, we found that anterograde-labelled nasal axons frequently arborized in the inappropriate region(s) of the rostral tectum (7 of 23 embryos), in addition to the caudal area (Fig. 4C). In some cases, axonal trajectories were deflected from their normal tectal path (Fig. 4Cb), although they finally connected to the caudal area (8 of 23 embryos). Temporal axons made normal connections (21 embryos). In control embryos injected with the alkaline phosphatase ($n = 22$) or CBF-1 ($n = 22$) virus, all nasal axons behaved normally.

The distorted trajectories and aberrant projections in these embryos indicate that these transcription factors probably control expression of the receptors on the retinal axons that detect position-dependent cues in the tectum. However, normal connections were also observed in both cases. This may have been because not all of the cells became infected with the retrovirus or other factors, including endogenous CBF-1 or CBF-2, counteract the effect of ectopic expression, or because ectopic expression occurred too late to shift the projection fate. Temporal, but not nasal, retinal axons specifically recognize and avoid a repulsive component in the posterior tectal membranes *in vitro*¹⁸. Provided that CBF-1 and CBF-2 are a transcriptional repressor¹⁹ and activator, respectively, it is conceivable that CBF-1 downregulates and CBF-2 activates expression of the receptor molecule for the repulsive factor.

Since formulation of the chemoaffinity hypothesis, several position-specific molecules have been discovered along the rostrocaudal axis of the retina or tectum^{20–26}, but their functions *in vivo* have not been revealed. The rostrocaudal displacement of connections caused by mixexpression of CBF-1 or CBF-2 indicates that these transcriptional regulators control the positional identity in retinal ganglion cells along the nasotemporal axis. Recently, the tectal map of chicken was perturbed by misexpression of *en*, the vertebrate homologue of *Drosophila engrailed*²⁷. Our methods allow a testing of whether the misexpressed transcription factors change the topographical expression of other molecules. We could not detect alteration in the graded messenger RNA expression of *Cek4* (*Mek4*)²² in the retina infected extensively with CBF-1 or CBF-2 virus (not shown), but it is possible that the number of switched cells was too small to be detected by *in situ* hybridization. Our findings provide evidence of the chemoaffinity theory, and may allow identification of other topographic molecules involved in the target-sensing mechanism. □

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1. Udin, S. B. & Fawcett, J. W. *Annu. Rev. Neurosci.* **11**, 289–327 (1988).
2. Sanes, J. R. *Curr. Opin. Neurobiol.* **3**, 67–74 (1993).
3. Kaprielian, Z. & Patterson, P. H. *BioEssays* **16**, 1–11 (1994).
4. Sperry, R. W. *Proc. Natl Acad. Sci. U.S.A.* **50**, 703–710 (1963).
5. Lai, E., Clark, K. L., Burley, S. K. & Darnell, J. E. *Proc. Natl Acad. Sci. U.S.A.* **90**, 10421–10423 (1993).
6. Tao, W. & Lai, E. *Neuron* **8**, 957–966 (1992).
7. Chang, H. W., Li, J., Kretschmar, D. & Vogt, P. K. *Proc. Natl Acad. Sci. U.S.A.* **92**, 447–451 (1995).
8. Li, J. & Vogt, P. K. *Proc. Natl Acad. Sci. U.S.A.* **90**, 4490–4494 (1993).
9. Hatini, V., Tao, W. & Lai, E. *J. Neurobiol.* **25**, 1293–1309 (1994).
10. Hamburger, V. & Hamilton, H. L. *J. Morphol.* **88**, 49–92 (1951).
11. Mey, J. & Thanos, S. *J. Hirnforsch.* **33**, 673–702 (1992).
12. Dütting, D. & Thanos, S. *Dev. Biol.* **167**, 263–281 (1995).
13. Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. & Sutcliffe, P. J. *Virology* **61**, 3004–3012 (1987).
14. Morgan, B. A. & Fekete, D. M. *Methods Cell Biol.* **51**, 185–218 (1996).
15. Homburger, S. A. & Fekete, D. M. *Dev. Dyn.* **206**, 112–120 (1996).
16. Thanos, S. & Bonhoeffer, F. *J. Comp. Neurol.* **261**, 155–164 (1987).
17. Nakamura, H. & O'Leary, D. D. *J. Neurosci.* **9**, 3776–3795 (1989).
18. Walter, J. M., Henke-Fahle, S. & Bonhoeffer, F. *Development* **101**, 909–913 (1987).
19. Li, J., Chang, H. W., Lai, E., Parker, E. J. & Vogt, P. K. *Cancer Res.* **55**, 5540–5544 (1995).
20. McLoon, S. C. *J. Neurosci.* **11**, 1470–1477 (1991).
21. Deitcher, D. L., Fekete, D. M. & Cepko, C. L. *J. Neurosci.* **14**, 486–498 (1994).
22. Cheng, H.-J., Nakamoto, M., Bergemann, A. D. & Flanagan, J. G. *Cell* **82**, 371–381 (1995).
23. Savitz, J. M., Trisler, D. & Hill, D. C. *Neuron* **14**, 253–261 (1995).
24. Itasaki, N., Ichijo, H., Hama, C., Matsuno, T. & Nakamura, H. *Development* **113**, 1133–1144 (1991).
25. Drescher, U. et al. *Cell* **82**, 359–370 (1995).
26. Stahl, B., Müller, B., von Boxberg, Y., Cox, E. C. & Bonhoeffer, F. *Neuron* **5**, 733–743 (1990).
27. Itasaki, N. & Nakamura, H. *Neuron* **16**, 55–62 (1996).
28. Wang, Z. & Brown, D. D. *Proc. Natl Acad. Sci. U.S.A.* **88**, 11505–11509 (1991).
29. Schwartz, R. M. & Dayhoff, M. O. in *Atlas of Protein Sequence and Structure* (ed. Dayhoff, M. O.) 353–358 (National Biomedical Research Foundation, Washington DC, 1979).

30. Wilkinson, D. G. in *In situ Hybridization, a Practical Approach* (ed. Wilkinson, D. G.) 1–83 (IRL, Oxford, 1992).

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CORRESPONDENCE and requests for materials should be addressed to M.N. (e-mail: madon@nibb.ac.jp).

Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1

Takashi Nagasawa*†, Seiichi Hirota‡, Kazunobu Tachibana*, Nobuyuki Takakura§, Shin-ichi Nishikawa§, Yukihiko Kitamura‡, Nobuaki Yoshida*, Hitoshi Kikutani† & Tadimitsu Kishimoto||

* Department of Immunology, Research Institute, Osaka Medical Center for Maternal and Child Health, 840 Murodo-cho, Izumi, Osaka 590-02, Japan

† Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565, Japan

‡ Department of Pathology, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565, Japan

§ Department of Molecular Genetics, Faculty of Medicine, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606, Japan

|| Department of Medicine III, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565, Japan

THE chemokines are a large family of small, structurally related cytokines^{1,2}. The physiological importance of most members of this family has yet to be elucidated, although some are inducible inflammatory mediators that determine leukocyte chemotaxis^{1–5}. Pre-B-cell growth-stimulating factor/stromal cell-derived factor-1 (PBSF/SDF-1) is a member of the CXC group of chemokines^{6,7}. PBSF/SDF-1 stimulates proliferation of B-cell progenitors *in vitro*⁶ and is constitutively expressed in bone-marrow-derived stromal cells^{6,7}. Here we investigate the physiological roles of PBSF/SDF-1 by generating mutant mice with a targeted disruption of the gene encoding PBSF/SDF-1. We found that mice lacking PBSF/SDF-1 died perinatally and that although the numbers of B-cell progenitors in mutant embryos were severely reduced in fetal liver and bone marrow, myeloid progenitors were reduced only in the bone marrow but not in the fetal liver, indicating that PBSF/SDF-1 is responsible for B-cell lymphopoiesis and bone-marrow myelopoiesis. In addition, the mutants had a cardiac ventricular septal defect. Hence, we have shown that the chemokine PBSF/SDF-1 has several essential functions in development.

The PBSF/SDF-1 gene was inactivated in exon 2 which encodes amino acids 2 to 41 and contains three cysteine residues and the presumptive receptor-binding region of CXC chemokines⁸ (Fig. 1).

Homozygous mutant embryos were present at the expected ratios until day 15.5 of embryogenesis (E15.5). However, about half the PBSF/SDF-1^{−/−} embryos were dead at E18.5; PBSF/SDF-1^{−/−} neonates died within an hour. Viable PBSF/SDF-1^{−/−} embryos appeared to be macroscopically normal, although most PBSF/SDF-1^{−/−} embryos were slightly reduced in size and weight from E13.5.

To investigate the effect of the PBSF/SDF-1 mutation on a

haematopoiesis, we examined the fetal liver and bone marrow. The liver is the principal haematopoietic organ from ~E11.5–12.5 until the first postnatal week. Histological examination of the E18.5 PBSF/SDF-1^{-/-} fetal liver revealed no obvious abnormalities. Flow cytometry analysis of fetal liver cell suspension was done to characterize the effect of the PBSF/SDF-1 mutation on the development of B lymphocytes, monocytes and granulocytes. B220⁺ cells were first detected at significant levels (1–2%) in the fetal liver at E15.5–16.5 (not shown)⁹. In the E18.5 PBSF/SDF-1^{+/+} (Fig. 2a) and heterozygous (+/–) fetal livers (not shown), pro-B (B220⁺ CD43⁺) and pre-B (B220⁺ CD43[–]) cells were readily detected¹⁰ (4.6% and 5.3%, respectively). However, the numbers of these cells were severely reduced in the mutant embryos at 0.4% and 0.2%, respectively (Fig. 2a). The numbers of granulocytes (Gr-1⁺) and monocytes (CD11b⁺ CD18⁺) in E18.5 mutants were unaltered (Fig. 2a). The effect of the PBSF/

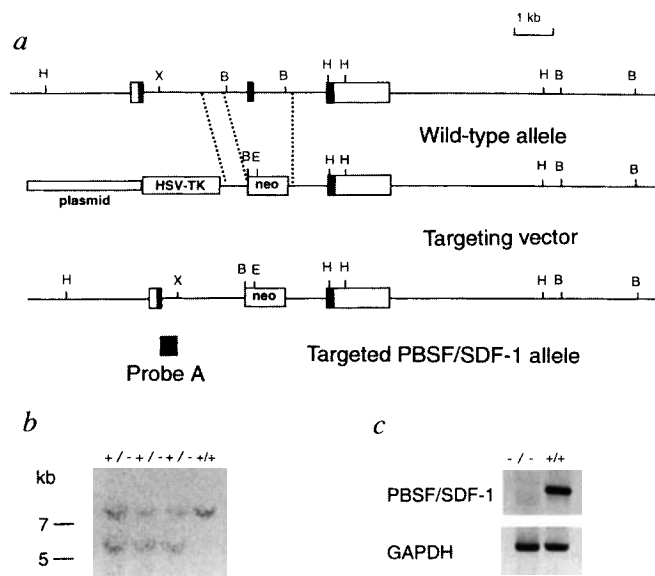


FIG. 1. Targeting of the PBSF/SDF-1 gene. **a**, From the top, diagrams depict the structures of the PBSF/SDF-1 gene, the targeting vector and the mutated allele. The coding regions of the gene are indicated as filled boxes; empty boxes represent the 5' and 3' untranslated regions. Dotted lines delineate the homologous fragments used in the targeting vector. Probe A is the external probe for Southern hybridization (**b**). Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xho*I. **b**, Representative Southern blot analysis of DNA samples from non-recombinant and recombinant colonies. The 7-kb wild-type and 5-kb targeted allele *Eco*RI and *Hind*III fragments identified by probe A are indicated. **c**, Reverse transcription (RT)-PCR analysis of the mRNA samples from E16.5 embryos. Total RNA was amplified with PBSF/SDF-1-specific primers that bracket exon 2. RT-PCR amplification was also done for the ubiquitously expressed GAPDH mRNA as a control for the presence of amplifiable RNA.

METHODS. Genomic DNA containing the PBSF/SDF-1 locus was isolated from a library of mouse strain 129 DNA (Stratagene). Exon 2 of the PBSF/SDF-1 gene was replaced by the *neo* gene and the herpes simplex thymidine kinase gene was fused at the 5' end. The targeting vector was inserted in E14.1 cells by electroporation and homologous recombination events were enriched by selection with G418 and gancyclovir and identified by PCR. The structure of the mutant locus and the presence of a single insertion in ES cell colonies were verified by Southern hybridization. Two independently mutated ES colonies were used to produce mutant mice by blastocyst injection as described²³. In **b**, DNAs prepared from ES cells were digested with *Eco*RI and *Hind*III, transferred to a nylon membrane and then hybridized with the 500-bp probe A that flanked the 5' homology region. In **c**, RT-PCR was performed according to standard protocols, starting with ~5 µg total RNA isolated from whole mouse embryos. PCR was carried out for 40 cycles. 539-bp products were amplified using PBSF/SDF-1-specific primers. Primer sequences were as follows: forward, PBSF/SDF-1, 5'-ACGCCAAGGTCGTCGCTGCTGG-3'; reverse, PBSF/SDF-1, 5'-GTAGGGTAATACAATTCCTTAGA-3'.

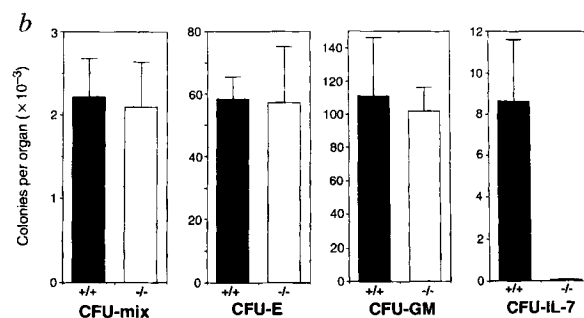
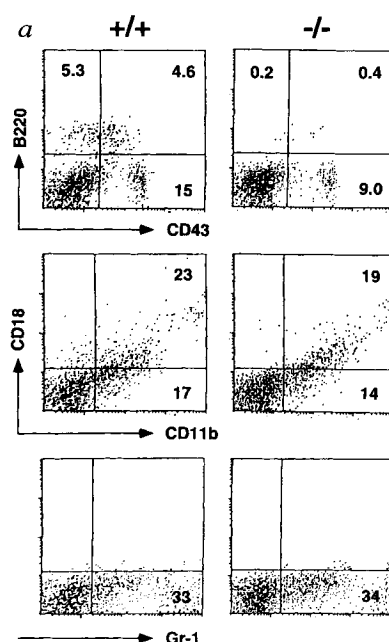


FIG. 2 Defects of B-cell lymphopoiesis in PBSF/SDF-1^{-/-} fetal liver. Cells obtained from the fetal livers of E18.5 PBSF/SDF-1^{+/+} or size-matched -/- embryos were analysed by flow cytometry or by *in vitro* colony assays. **a**, Flow cytometry analysis. The total numbers of nucleated cells recovered from the fetal livers of PBSF/SDF-1^{+/+} and -/- mice were $(2.9 \pm 0.2) \times 10^7$ and $(2.7 \pm 0.2) \times 10^7$, respectively. Top, fetal liver cells stained with antibodies against B220 (FITC) or against CD43 (PE). Middle, fetal liver cells stained with antibodies against CD11b (PE) or CD18 (FITC). Bottom, fetal liver cells stained with antibodies to Gr-1 (PE). The percentages of cells with a particular cell-surface expression phenotype are indicated within the appropriate quadrant. **b**, Haematopoietic progenitor colony assays. CFU-mix, CFU-E, CFU-GM and CFU-IL-7. Results from four embryos from three litters are shown.

METHODS. **a**, Flow cytometry analysis was done on single-cell suspensions of fetal liver. Cells were stained with antibodies using standard procedures. The following antibody conjugates were used: fluorescein (FITC)-B220, phycoerythrin (PE)-CD43, FITC-CD18, biotin-CD11b, biotin-Gr-1 (Pharmingen). Biotinylated antibodies were detected with PE-streptavidin (Pharmingen). Fluorescence was analysed on a FACStar flow cytometer (Beckton-Dickinson). Adult mouse spleen and bone marrow suspension were used as positive controls in setting staining gates. Top, only cells falling within the lymphocyte scatter gate are shown. The percentage of cells within the gate from PBSF/SDF-1^{+/+} and -/- mice was $60.2 \pm 0.1\%$ and $64.9 \pm 1.4\%$, respectively. **b**, Methylcellulose culture was performed as described²⁴. Fetal liver cells were incubated in 1 ml of culture medium containing alpha medium (GIBCO), 1.2% methylcellulose (Muromachi Kagaku Kogyo), 30% FCS, 1% deionized BSA (Sigma), 50 µM 2-mercaptoethanol and haematopoietic factors: 200 U ml⁻¹ recombinant murine IL-3 (ref. 24) and 2 U ml⁻¹ recombinant human Epo (Chugai) or 20 U ml⁻¹ murine recombinant IL-7 (from T. Sudo). Colony formation was monitored at 4 days (CFU-E) and 7 days (CFU-mix, CFU-GM and CFU-IL-7) after inoculation. The numbers of colonies per fetal liver are indicated.

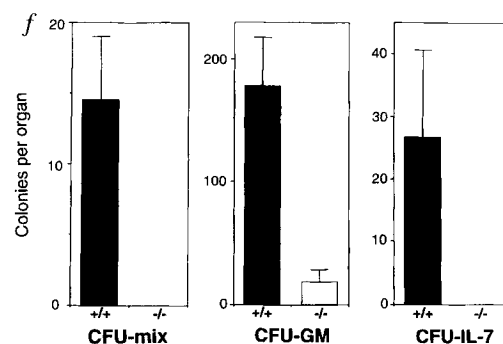
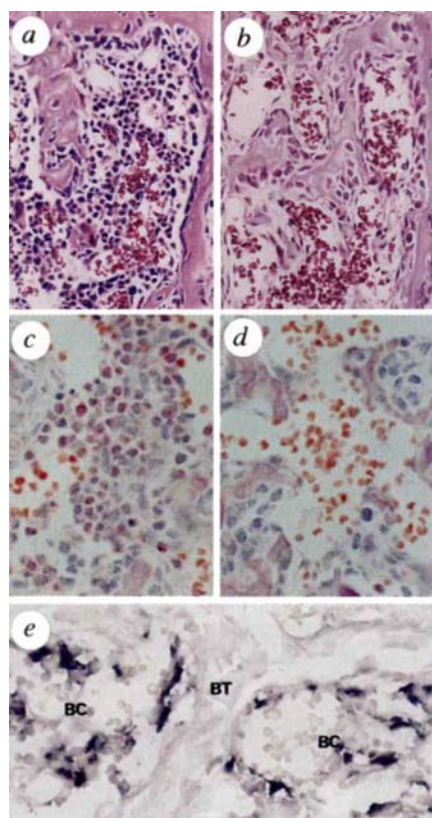


FIG. 3 Defects of myelopoiesis and B-cell lymphopoiesis in PBSF/SDF-1^{-/-} bone marrow. Histological analysis of bone marrow in E18.5 PBSF/SDF-1^{+/+} (a, c) and size-matched -/- embryos (b, d). a–d, Sections through the femurs. a, b, Sections were stained with haematoxylin–eosin (×238). c, d, Sections were stained for chloroacetyl esterase activity (×400). Chloroacetyl-esterase-expressing cells have a red cytoplasm. Almost all nucleated cells in b and d are osteoblastic cells. e, Expression of PBSF/SDF-1 mRNA in E18.5 wild-type bone marrow (×400). BT, bone trabeculae; BC, bone-marrow cavity. f, Haematopoietic progenitor colony assays of E18.5 wild-type and homozygous bone marrows. CFU-mix, CFU-GM and CFU-IL-7. Results from four embryos from three litters are shown.

METHODS. a–d, Tissues were fixed in 4% paraformaldehyde and processed into paraffin sections by routine procedures. For c and d, cytochemistry for chloroacetyl esterase was done on the sections using naphthol AS-D chloroacetate (Sigma) as substrate. For e, *in situ* hybridizations were done using digoxigenin-labelled probes as described²⁵. The PBSF/SDF-1 probe was generated by antisense transcription of the cDNA fragment. For f, bilateral femurs of E18.5 embryos were disrupted to yield a single-cell suspension. Methylcellulose culture was done as for Fig. 2b. Numbers of colonies per two femurs are indicated.

SDF-1 mutation on haematopoietic progenitors in E18.5 fetal liver was also investigated using *in vitro* colony assays. PBSF/SDF-1^{-/-} fetal livers yielded normal numbers of erythroid–myeloid mixed colonies (CFU-mix), erythroid colonies (CFU-E) and myeloid colonies (CFU-GM), but greatly reduced numbers of pre-B-cell colonies (CFU-IL-7) (Fig. 2b). These results indicate that the effect of PBSF/SDF-1 mutation in the fetal liver is B-lymphoid-specific. The decline of B cells in the mutant fetal livers was apparent already at E16.5 (not shown).

The final haematopoietic station during fetal development is the bone marrow, the definitive adult haematopoietic site. In mouse bone marrow, active B-cell lymphopoiesis and myelopoiesis appear from E17 (refs 11–13). Histological examination of serial long-bone sections revealed that a large number of haematopoietic cells, most of which were developing granulocytic cells, contained chloroacetyl esterase activity which could be easily identified in the bone-marrow cavities of control embryos from E17.5 (Fig. 3a, c). To our surprise, these cells were virtually absent from E18.5 mutant embryos (Fig. 3b, d). The structure of cortical bones and trabeculae, the formation of bone-marrow cavities, and the number of osteoblastic cells lining the cortical bone and trabeculae were normal even in the mutants (Fig. 3a–d). The identical phenotype was seen in other bones, for example, the

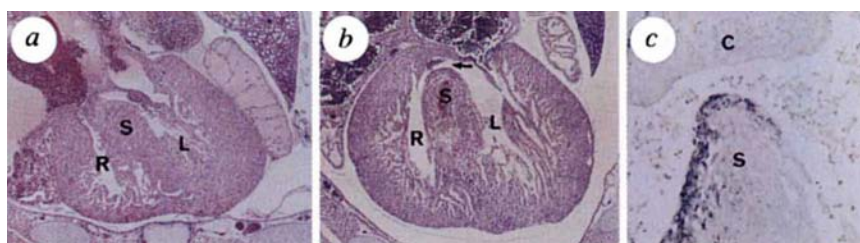
bones of the rib and vertebral column (not shown). *In vitro* colony assays were used to compare the numbers of haematopoietic progenitors in E18.5 bone marrows from all genotypes. The numbers of CFU-mix, CFU-GM and CFU-IL-7 were greatly reduced in the PBSF/SDF-1^{-/-} bone marrow (Fig. 3f). Mutation of PBSF/SDF-1 eliminated myelopoiesis as well as B-cell lymphopoiesis in the bone marrow. *In situ* hybridization in the E18.5 wild-type bone marrow showed that PBSF/SDF-1 messenger RNA was expressed in spindle-shaped stromal cells that could be distinguished from osteoblastic cells and haematopoietic cells (Fig. 3e), supporting a role for PBSF/SDF-1 in the bone marrow.

Thymuses of E18.5 PBSF/SDF-1^{-/-} embryos were histologically normal and contained normal numbers of T-cell progenitors, as monitored by flow cytometry analysis with CD4 and CD8 in comparison with control littermates (not shown).

Histological analysis of other organs revealed abnormal cardiac development in PBSF/SDF-1^{-/-} embryos. PBSF/SDF-1^{-/-} embryos had a defect of the membranous portion of the ventricular septum at E13.5–18.5 (Fig. 4b) compared with control embryos (Fig. 4a). The aorta and pulmonary artery outflow tracts and valves were normal. *In situ* hybridization revealed that PBSF/SDF-1 mRNA was expressed in the endocardium of the muscular ventricular septum at E12.5, when the membranous

FIG. 4 Ventricular septal defects in PBSF/SDF-1^{-/-} embryos. Haematoxylin–eosin staining of transverse sections of heart in E18.5 PBSF/SDF-1^{+/+} (a) and size-matched -/- embryos (b) (×20). Arrow in b indicates a ventricular septal defect. c, Expression of PBSF/SDF-1 mRNA in E12.5 wild-type heart (×100). L, left ventricle; R, right ventricle; S, interventricular septum; C, endocardial cushion.

METHODS. Tissues were fixed and processed as for Fig. 3a and b (a, b in this figure) and Fig. 3e (c).



portion of the ventricular septum will soon be formed, suggesting a role of PBSF/SDF-1 in ventricular septum formation (Fig. 4c). Dying PBSF/SDF-1^{-/-} neonates also had a ventricular septal defect but did not have an enlarged left heart or oedematous lungs (not shown), so the cardiac defect may not be the cause of their death.

Our data indicate that PBSF/SDF-1 has several critical functions in development. First, PBSF/SDF-1 is an essential cytokine for B-cell lymphopoiesis during embryonic development. Interleukin(IL)-7 was the first example of a cytokine that is essential for B-cell lymphopoiesis *in vivo*¹⁴⁻¹⁶. Although disruption of IL-7 affects expansion of pre-B cells but not of pro-B cells¹⁶, the numbers of both pro-B and pre-B cells were reduced in PBSF/SDF-1 deficient mice because of an earlier developmental defect. Mice carrying mutations in several transcription factor genes generate no, or very few, B-cell progenitors¹⁷⁻¹⁹. Such factors may be involved in the expression of an unidentified PBSF/SDF-1 receptor or signalling through it. Second, PBSF/SDF-1 is required for myelopoiesis in the bone marrow but not in the fetal liver. Haematopoietic precursors migrate from other sites of haematopoiesis and colonize the bone marrow during embryogenesis. Although the contribution of cytokines in this process has yet to be elucidated, our results raise the possibility that stromal-cell-derived PBSF/SDF-1 may support colonization of the bone marrow by haematopoietic precursors as a microenvironmental factor. Finally, PBSF/SDF-1 is responsible for heart ventricular septum formation, indicating that PBSF/SDF-1 function is not limited to the haematopoietic system. The same ventricular septum defect was also observed in mice deficient in endothelin-1 or RXR- α (refs 20-22). Whether and how PBSF/SDF-1, endothelin-1 and retinoic acid cooperate in the formation of the

ventricular septum is unknown, but all these soluble factors are required.

PBSF/SDF-1 is therefore the first example of a chemokine that has an essential developmental function, suggesting that chemokines may be important in other roles apart from inducing chemotaxis in inflammation. □

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1. Oppenheim, J. et al. *A. Rev. Immun.* **9**, 617-648 (1991).
2. Kunkel, S. et al. *Immun. Today* **16**, 559-561 (1995).
3. Cacalano, G. et al. *Science* **265**, 682-684 (1994).
4. Moore, M. et al. *Science* **269**, 1591 (1995).
5. Cook, D. et al. *Science* **269**, 1583-1585 (1995).
6. Nagasawa, T. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 2305-2309 (1994).
7. Tashiro, K. et al. *Science* **261**, 600-603 (1993).
8. Clark-Lewis, I. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 3574-3577 (1993).
9. Hardy, R. & Hayakawa, K. *Proc. natn. Acad. Sci. U.S.A.* **88**, 11550-11554 (1991).
10. Hardy, R. et al. *J. exp. Med.* **173**, 1213-1225 (1991).
11. Ogawa, M. et al. *EMBO J.* **7**, 1337-1343 (1988).
12. Tavassoli, M. *Blood Cells* **1**, 269-281 (1991).
13. Delassus, S. & Cumano, A. *Immunity* **4**, 97-106 (1996).
14. Grabstein, K. et al. *J. exp. Med.* **178**, 257-264 (1993).
15. Peshon, J. et al. *J. exp. Med.* **180**, 1955-1960 (1994).
16. Freedman-Jeffrey, U. et al. *J. exp. Med.* **181**, 1519-1526 (1995).
17. Georgopoulos, K. et al. *Cell* **79**, 143-156 (1994).
18. Zhuang, Y. et al. *Cell* **79**, 875-884 (1994).
19. Scott, E. W. et al. *Science* **265**, 1573-1577 (1994).
20. Kurihara, Y. et al. *J. clin. Invest.* **96**, 293-300 (1995).
21. Sucov, H. et al. *Genes Dev.* **8**, 1007-1018 (1994).
22. Kastner, P. et al. *Cell* **78**, 987-1003 (1994).
23. Tanaka, T. et al. *Cell* **80**, 353-361 (1995).
24. Ogawa, M. et al. *Development* **117**, 1089-1098 (1993).
25. Hirota, S. et al. *Molec. Brain Res.* **15**, 47-54 (1992).

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CORRESPONDENCE and requests for materials should be addressed to T.N. (e-mail: immunol@osk.threewebnet.or.jp).

Functional interaction of β -catenin with the transcription factor LEF-1

Jürgen Behrens*, Jens P. von Kries*, Michael Kühl†, Laurakay Bruhn‡, Doris Wedlich†, Rudolf Grosschedl‡ & Walter Birchmeier*

* Max Delbrück Centre for Molecular Medicine, Robert-Rössle-Strasse 10, 13122 Berlin, Germany

† University of Ulm, Albert Einstein Allee 11, 89081 Ulm, Germany

‡ Howard Hughes Medical Institute, and Departments of Microbiology and Biochemistry, University of California, San Francisco, California 94143-0414, USA

THE cytoplasmic proteins β -catenin of vertebrates and armadillo of *Drosophila* have two functions: they link the cadherin cell-adhesion molecules to the cytoskeleton¹⁻⁴, and they participate in the wnt/wingless signalling pathway⁵⁻⁷. Here we show, in a yeast two-hybrid screen, that the architectural transcription factor LEF-1 (for lymphoid enhancer-binding factor)⁸⁻¹⁰ interacts with β -catenin. In mammalian cells, coexpressed LEF-1 and β -catenin form a complex that is localized to the nucleus and can be detected by immunoprecipitation. Moreover, LEF-1 and β -catenin form a ternary complex with DNA that displays an altered DNA bend. Microinjection of LEF-1 into *Xenopus* embryos induces axis duplication, which is augmented by interaction with β -catenin. Thus β -catenin regulates gene expression by direct interaction with transcription factors such as LEF-1, providing a molecular mechanism for the transmission of signals from cell-adhesion components or wnt protein to the nucleus.

To find a link between cell-adhesion molecules and the nucleus

we searched for new interaction partners of β -catenin, a member of the family of armadillo repeat proteins. In a yeast two-hybrid screen¹¹, several cDNA clones were identified, one of which encodes the first 76 amino acids of the transcription factor LEF-1 (refs 8-10) (Fig. 1A). This protein did not interact with the adenomatous polyposis coli (APC) gene product, p120^{CAS} or importin α , which are other armadillo repeat proteins⁵ demonstrating specificity of the interaction (data not shown). Full-length LEF-1 and a mutant protein lacking the high-mobility group (HMG) (DNA-binding) domain also interacted with β -catenin, but amino-terminal truncations of LEF-1 did not (Fig. 1A). The interaction domain in β -catenin was delineated to the region of the 13 armadillo repeats, with the N-terminal repeats 1-7 being partly sufficient for interaction with LEF-1 (Fig. 1B). The interaction between LEF-1 and β -catenin was also demonstrated by co-immunoprecipitation of LEF-1 with anti- β -catenin antibodies from transiently transfected mammalian cells (Fig. 1C); β -catenin could also be co-immunoprecipitated with anti-LEF-1 antibodies (data not shown). Together, these data show that the N-terminal domain of LEF-1 is necessary and sufficient for the interaction with β -catenin.

To determine whether the association of β -catenin with LEF-1 results in colocalization of this complex in either the nucleus or cytoplasm, we transiently transfected expression constructs encoding these proteins into mammalian (Neuro 2A) cells, and visualized the proteins by indirect immunofluorescence. Only β -catenin was detected in both the cytoplasm and nucleus (Fig. 1Da; note that Neuro 2A cells lack endogenous cadherins). In contrast, co-expression with LEF-1 resulted in complete translocation of β -catenin to the nucleus (Fig. 1Db) and colocalization with LEF-1 (Fig. 1Dc). The nuclear translocation of β -catenin was dependent both on armadillo repeats 1-7 and the N-terminal domain of LEF-1 (Fig. 1Dd, e). Deletion of the HMG domain of LEF-1 prevented nuclear localization, consistent with the identification of a nuclear localization sequence in this domain¹². LEF-1 also

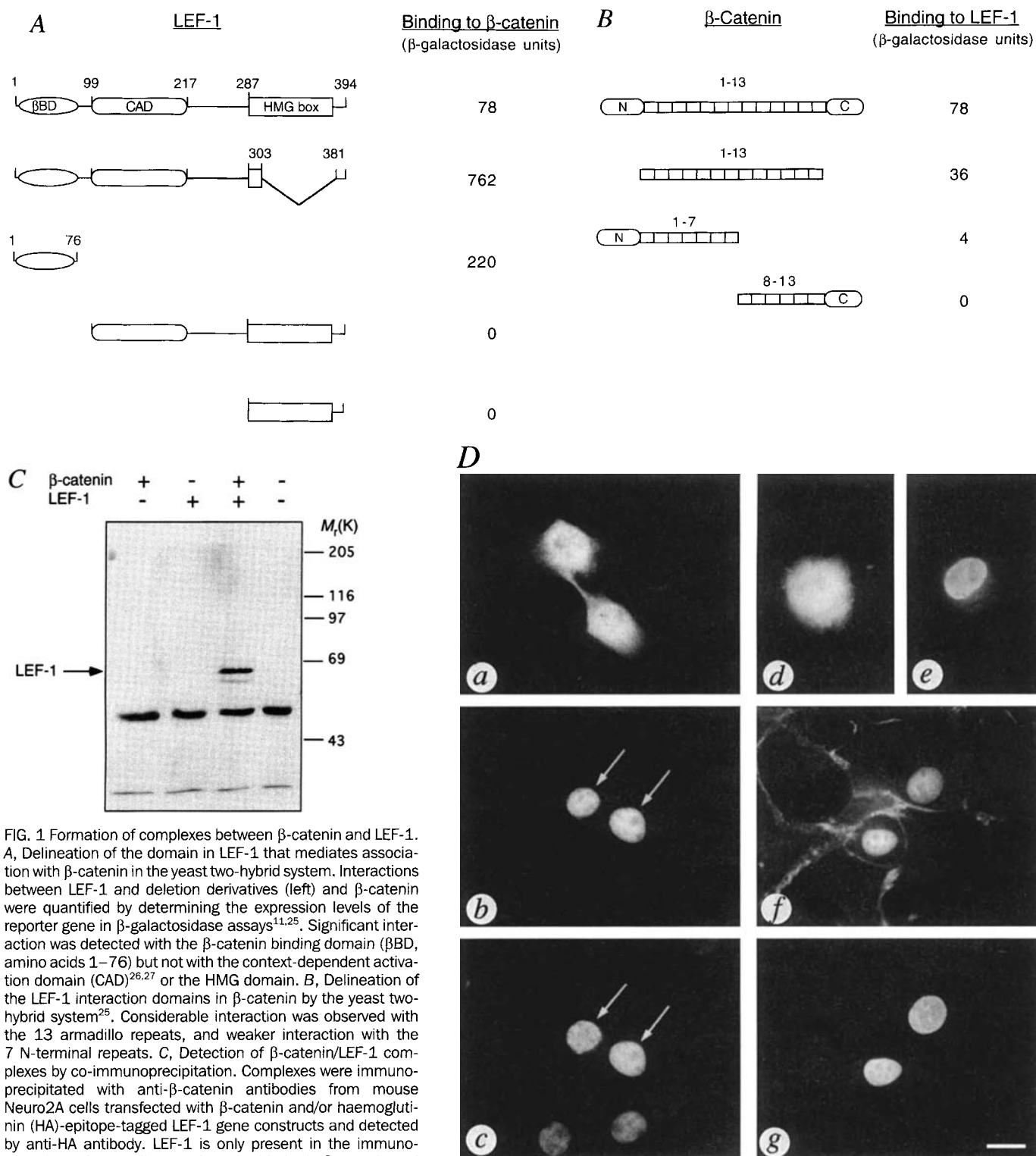


FIG. 1 Formation of complexes between β -catenin and LEF-1. **A**, Delineation of the domain in LEF-1 that mediates association with β -catenin in the yeast two-hybrid system. Interactions between LEF-1 and deletion derivatives (left) and β -catenin were quantified by determining the expression levels of the reporter gene in β -galactosidase assays^{11,25}. Significant interaction was detected with the β -catenin binding domain (β BD, amino acids 1–76) but not with the context-dependent activation domain (CAD)^{26,27} or the HMG domain. **B**, Delineation of the LEF-1 interaction domains in β -catenin by the yeast two-hybrid system²⁵. Considerable interaction was observed with the 13 armadillo repeats, and weaker interaction with the 7 N-terminal repeats. **C**, Detection of β -catenin/LEF-1 complexes by co-immunoprecipitation. Complexes were immunoprecipitated with anti- β -catenin antibodies from mouse Neuro2A cells transfected with β -catenin and/or haemagglutinin (HA)-epitope-tagged LEF-1 gene constructs and detected by anti-HA antibody. LEF-1 is only present in the immunoprecipitate from cells transfected with both β -catenin and LEF-1. Right, molecular weight markers (Sigma). **D**, Sub-cellular localization of LEF-1 and β -catenin in transfected Neuro2A (**a–e**) and MDCK (**f, g**) cells. **a**, Without LEF-1, transfected β -catenin is detected by indirect immunofluorescence¹ in both cytoplasm and nucleus of Neuro2A cells. **b, c**, With LEF-1, β -catenin is localized exclusively to the nucleus, as shown by double-labelling immunofluorescence with anti- β -catenin (**b**) and anti-HA (**c**) antibodies. Nuclear localization of β -catenin and LEF-1 in identical cells is marked by arrows. **d, e**, With truncated LEF-1, lacking the β -catenin binding domain, β -catenin is distributed throughout the cell (**d**), whereas LEF-1 is found in the nucleus (**e**). **f, g**, In MDCK epithelial cells transfected with LEF-1, endogenous β -catenin is present in both the nucleus and at the cell membranes (**f**), whereas LEF-1 is exclusively in the nucleus (**g**). Scale bar, 10 μ m.

METHODS. For the initial yeast two-hybrid screen, the region encoding the

13 armadillo repeats of β -catenin was fused to the GAL4 DNA-binding domain in pGBT9 (Clontech) and assayed for interaction with proteins encoded by a VP16 activation-domain cDNA library from embryonic day 10.5 (E10.5) mouse embryos. Interactions were detected in the yeast strain HF7c on media lacking histidine and containing 25 mM aminotriazole. Full-length LEF-1 and deletion derivatives, generated by restriction digest or polymerase chain reaction (PCR) were fused to the C terminus of the GAL4 DNA-binding domain and assayed for interaction with β -catenin fused to the GAL4 activation domain in pGAD424 (Clontech) in a quantitative β -gal assay²⁵. In the two-hybrid screen, other known interaction partners of β -catenin were detected, such as several cadherins (E-, N-, K-, OB-, VE-cadherins) and APC, and clones encoding desmoglein 2 and cytokeratin 18.

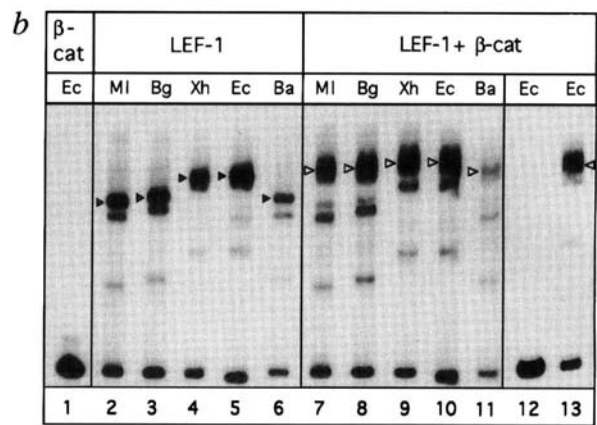
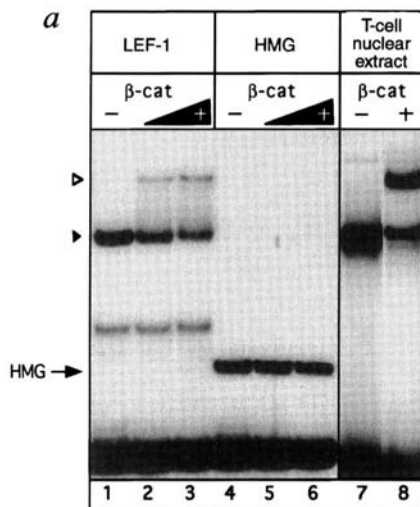
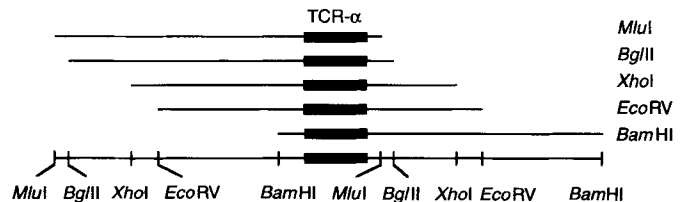


FIG. 2 DNA-binding properties of the β -catenin/LEF-1 complex. **a**, LEF-1/ β -catenin/DNA ternary complex formation in an electrophoretic mobility-shift assay. Full-length LEF-1 protein (25 ng, 0.5 pmol; lanes 1–3) or 5 ng (0.5 pmol) HMG domain of LEF-1 (lanes 4–6) were incubated alone or with 10 ng (0.1 pmol) or 30 ng (0.3 pmol) β -catenin and a radiolabelled oligonucleotide containing a LEF-1 binding site. Nuclear extracts (1 μ g) from Jurkat cells expressing endogenous LEF-1 were incubated with or without 100 ng β -catenin (lanes 7, 8). The LEF-1/ β -catenin/DNA complex is indicated by an open triangle and the LEF-1/DNA complex by a filled triangle. **b**, Circular permutation analysis of DNA bending induced by LEF-1 (100 ng, 2 pmol) alone and in combination with β -catenin (100 ng, 1 pmol). Lanes 2–6, LEF-1/DNA complexes (filled triangles); Lanes 7–11, LEF-1/ β -catenin/DNA complexes (open triangles); Lane 1, β -catenin and DNA; lanes 12, 13, competition of the LEF-1/ β -catenin/DNA complexes with specific and nonspecific DNA, respectively. The restriction enzymes (Ec, EcoRV; Ml, MluI; Bg, BglII; Xh, XhoI; Ba, BamHI) were used to generate the circularly permuted DNA probes from the pBend2 plasmid²⁸ in which the LEF-1 site (filled box) is inserted between a duplicated polylinker. In the DNA probes generated by digestion with the various enzymes, the LEF-1 binding site is located at different positions relative to the ends of the DNA fragment.



METHODS. His-tagged LEF-1 and β -catenin were expressed in *Escherichia coli* and purified to homogeneity by nickel chelate chromatography. LEF-1–HMG domain was purified, and electrophoretic mobility-shift assays were performed essentially as previously described¹⁴. DNA binding reactions contained 5 ng radiolabelled DNA fragment, 5–100 ng purified proteins, or 1 μ g Jurkat nuclear extract as indicated, and 50 ng poly(dI-dC) in 15 μ l binding buffer¹⁴. Radiolabelled DNA fragments: **a**, TCR- α enhancer fragment¹⁰; **b**, circularly permuted DNA fragments from pBend2 (ref. 28) containing the LEF-1 binding site. The extent of DNA bends in the circular permutation and helical phasing analyses were calculated by using the relative mobilities of the protein/DNA complexes with the maximal and minimal migration rates in algorithms described^{14,16}.

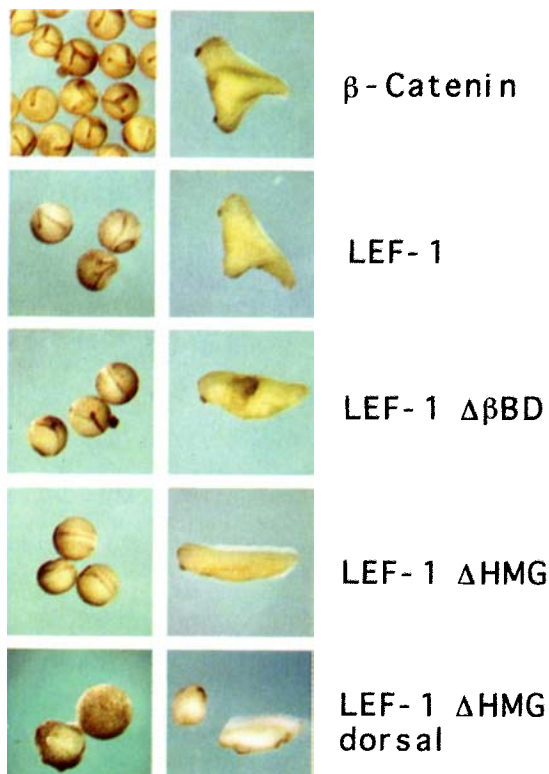


FIG. 3 Axis duplication in *Xenopus* embryos induced by microinjected LEF-1 and β -catenin mRNA. Embryos were examined at the neurula stage (left column) and at the tailbud stage (right column). Injection of 0.5 ng LEF-1 mRNA into ventral blastomeres induced secondary axes similar to those induced by 0.25 ng β -catenin mRNA. Injection of mRNA encoding a mutant form of LEF-1 lacking the β -catenin binding domain (Δ BBD) induced formation of only rudimentary secondary axes at a low frequency (see Table 1). No secondary axis formation was observed with LEF-1 lacking the HMG domain (Δ HMG). Injection of the Δ HMG mutant of LEF-1 into the dorsal blastomeres induced ventralization of the embryos which lacked anterior and dorsal structures (bottom). Ventralization was verified by *in situ* hybridization of the ventral marker β -globin. Wild-type and mutant LEF-1 were expressed in equal amounts, as indicated by western blotting. **METHODS.** RNA was produced by *in vitro* transcription using the pSP64T3 vector and SP6 RNA polymerase²⁹. RNAs were microinjected into the marginal zone of either of the two ventral or dorsal blastomeres of 4-cell-stage embryos. Injection and further cultivation of embryos were performed as described³⁰.

translocated coexpressed plakoglobin (γ -catenin), the closest relative of β -catenin^{1,13} into the nucleus (data not shown). To determine whether LEF-1 can translocate endogenously expressed β -catenin into the nucleus, we transfected the LEF-1 expression construct into epithelial (MDCK) cells. A large proportion of the endogenous β -catenin was detected in the nucleus, and the remaining fraction was located at the cell membrane presumably associated with E-cadherin (Fig. 1*Df, g*). Consistent with this observation, exogenous expression of E-cadherin in Neuro 2A cells, together with β -catenin and LEF-1, also resulted in β -catenin being attached to the plasma membrane without interfering with nuclear localization of LEF-1 (data not shown).

Previous data¹⁰ suggested that LEF-1 can function as an architectural transcription factor by inducing a sharp bend in the DNA helix, and by collaborating with other enhancer binding proteins. The association of β -catenin with LEF-1 in the nucleus suggested that the DNA-binding or regulatory properties of the transcription factor might be altered. Purified recombinant LEF-1 and β -catenin bind DNA as a ternary complex migrating at a slower rate in an electrophoretic mobility-shift assay than the LEF-1/DNA complex (Fig. 2*a*). In contrast, the HMG domain of LEF-1 showed no ternary complex formation with β -catenin. Addition of β -catenin to nuclear extracts from Jurkat T cells containing endogenous LEF-1 also led to the formation of a similar complex (Fig. 2*a*). To examine the effects of β -catenin on LEF-1-induced DNA bending, we performed a circular permutation analysis. Consistent with the previously determined LEF-1-induced DNA bend of $\sim 120^\circ$ (refs 14, 15), migration of LEF-1/DNA complexes was strongly dependent on the position of the binding site relative to the ends of the DNA fragments (Fig. 2*b*, lanes 2–6). In contrast, migration of ternary complexes formed in the presence of LEF-1 and β -catenin was significantly less dependent on the positions of the LEF-1 binding site, indicating that β -catenin decreases the extent of LEF-1-induced DNA bending (Fig. 2*b*, lanes 7–11). We calculated a decrease in the DNA bend angle of at least 40° . Given that circular permutation assays can be influenced by the shape of some DNA-binding proteins, we confirmed the change in DNA bending of LEF-1 in complex with β -catenin by a helical phasing analysis¹⁶ (data not shown). LEF-1-induced DNA bending can affect gene regulation¹⁰, so it was possible that β -catenin might affect these activities. Although transfection of β -catenin into T cells did not significantly contribute to LEF-1-mediated regulation of the T-cell receptor- α enhancer (data not shown), the association of β -catenin with LEF-1 might alter the regulatory properties of LEF-1 in other contexts. For example, LEF-1 has been shown to regulate epithelial-mesenchymal tissue interactions during organogenesis¹⁷, and the association with β -catenin may influence the role of LEF-1 and related transcription factors in embryonic development. Our data suggest that in these processes the inter-

TABLE 1 LEF-1-induced axis duplication in *Xenopus* embryos

Injected mRNA (ng)	Experiments	Embryos examined	Embryos with duplicated axis (% $\pm$ s.d.)
Ventral injection			
LEF-1 (0.5)	3	244	37.0 $\pm$ 2.3
LEF-1 (0.1)	2	102	11.8 $\pm$ 0.5
LEF-1 (0.05)	2	87	5.4 $\pm$ 2.7
LEF-1 $\Delta\beta$ BD (0.5)	3	193	6.4 $\pm$ 2.1
LEF-1 Δ HMG (0.5)	3	225	0.0 $\pm$ 0.0
β -catenin (0.25)	4	239	55.5 $\pm$ 8.9
β -catenin Δ CAR8–13 (1)	3	143	11.1 $\pm$ 2.4
β -catenin Δ NAR1–7 (1)	3	117	0.0 $\pm$ 0.0
β -catenin (0.05)	5	306	1.9 $\pm$ 1.7
β -catenin (0.05) + LEF-1 (0.5)	2	145	51.6 $\pm$ 1.6
β -catenin (0.05) + LEF-1 (0.1)	2	100	40.1 $\pm$ 4.8
β -catenin (0.05) + LEF-1 (0.05)	2	82	27.8 $\pm$ 8.3
β -catenin (0.05) + LEF-1 $\Delta\beta$ BD (0.5)	2	98	6.8 $\pm$ 4.4
β -catenin (0.05) + LEF-1 Δ HMG (0.5)	2	144	1.6 $\pm$ 1.6
β -catenin (0.05) + preprolactin (0.5)	3	231	1.1 $\pm$ 1.5
Dorsal injection			
LEF-1 Δ HMG (2)	3	94	90.1 $\pm$ 13.1 (1.2)
LEF-1 Δ HMG (4)	2	39	97.0 $\pm$ 3.0 (0.8)
preprolactin (2)	2	74	2.9 $\pm$ 0.9 (4.9)

Different concentrations of RNAs were microinjected into the marginal zone of either ventral or dorsal blastomeres as indicated in the first column (see Fig. 3). LEF-1 $\Delta\beta$ DB, deletion of the β -catenin binding domain; LEF-1 Δ HMG, deletion of the HMG domain (see Fig. 1*A*); β -catenin Δ CAR8–13, deletion of the C terminus and arm repeats 8–13; β -catenin Δ NAR1–7, deletion of the N terminus and repeats 1–7. Preprolactin RNA served as control. Embryos were scored for axis duplication at the neurula stage and for ventralization at the tailbud stage. The degree of ventralization is expressed as the average dorso-anterior index (DAI; ref. 7). Values of 5 and 0 represent normal and completely ventralized embryos, respectively; s.d., standard deviation.

action of the N terminus of LEF-1 with β -catenin alters the DNA-binding properties of the C-terminal HMG domain of LEF-1.

Previous studies suggested that β -catenin can induce the formation of a secondary body axis in early *Xenopus* embryos^{7,18–20}, other components of the wnt pathway also have this effect. To examine whether LEF-1 may be involved in this pathway, we microinjected LEF-1 messenger RNA into ventral blastomeres of *Xenopus* embryos. Formation of a secondary axis occurred readily, as was observed with β -catenin (Fig. 3 and Table 1). The LEF-1 induced axes, however, were less complete and lacked the most anterior structures, such as cement glands. Axis formation by LEF-1 was dependent on both the β -catenin interaction and HMG domains; LEF-1 lacking the β -catenin interaction domain produced a few rudimentary secondary axes, whereas none were induced by LEF-1 lacking the HMG domain (Fig. 3 and Table 1). We examined further whether the interaction between LEF-1 and β -catenin is required in this assay. Co-injection of suboptimal amounts of β -catenin and full-length LEF-1 mRNAs, but not mutant LEF-1 mRNAs, resulted in more axis duplication than was observed with the individual mRNAs put together (Table 1). Furthermore, injection of the LEF-1 mutant lacking the HMG domain into dorsal blastomeres resulted in ventralization of the *Xenopus* embryos (Fig. 3 and Table 1). This observation suggests that this particular mutant form of LEF-1 could act in a dominant-negative manner by interfering with the action of endogenous LEF-1 or a related family member in axis formation. Consistent

with this view, a recently isolated *Xenopus* homologue of LEF-1 is expressed in the oocyte and throughout gastrulation (M.K. and D.W., unpublished data). Together, these data indicate that LEF-1 cooperates with β -catenin in embryonic axis induction in *Xenopus*.

The members of the wnt/wingless pathway upstream of β -catenin/armadillo have been identified previously in both *Xenopus* and *Drosophila*^{5-7,18-23}. Genetic and biochemical analysis of this pathway indicated that the secreted proteins wnt-1/wingless, for example by binding to a member of receptor the *frizzled* family of receptors, transmit a signal to the cytoplasmic components dishevelled and zeste white-3/glycogen synthase kinase, which regulate the free cytoplasmic pool of armadillo/ β -catenin. The tumour-suppressor gene product APC also regulates β -catenin^{1,24}.

The molecular nature of components downstream of β -catenin is not known, although β -catenin has been suggested to function in the nucleus^{5,7}. The architectural transcription factor LEF-1 is now a strong candidate as a downstream target of β -catenin, given the observations that: (1) LEF-1 binds directly to β -catenin; (2) LEF-1 translocates β -catenin to the nucleus; (3) β -catenin modulates the DNA-binding properties of LEF-1; and (4) LEF-1, like β -catenin and members of the wnt pathway, induces axis duplication of *Xenopus* embryos. Although the genetic targets regulated by LEF-1 and β -catenin remain to be characterized, we have shown that LEF-1 is a new potential component of β -catenin-induced signal pathways, and may provide a link between cell-adhesion processes, the wnt/wingless pathway, and gene regulation. □

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- Hülsken, J., Birchmeier, W. & Behrens, J. *J. Cell Biol.* **127**, 2061–2069 (1994).
- Aberle, H. et al. *J. Cell Sci.* **107**, 3655–3663 (1994).
- Nagafuchi, A., Ishihara, S. & Tsukita, A. *J. Cell Biol.* **127**, 235–245 (1994).
- McCreary, P. D., Turck, C. W. & Gumbiner, B. *Science* **254**, 1359–1361 (1991).
- Peifer, M., Berg, S. & Reynolds, A. B. *Cell* **76**, 789–791 (1994).
- Noordermeer, J., Klingensmith, J., Perrimon, N. & Nusse, R. *Nature* **367**, 80–83 (1994).
- Funayama, N., Fagotto, F., McCreary, P. & Gumbiner, B. *J. Cell Biol.* **128**, 959–968 (1995).
- Travis, A., Amsterdam, A., Belanger, C. & Grosschedl, R. *Genes Dev.* **5**, 880–894 (1991).
- Waterman, M. L., Fischer, W. H. & Jones, K. *Genes Dev.* **5**, 656–669 (1991).
- Giese, K., Kingsley, C., Kirschner, J. R. & Grosschedl, R. *Genes Dev.* **9**, 995–1008 (1995).
- Fields, S. & Song, O. K. *Nature* **340**, 245–246 (1989).
- Prieve, M. G., Guttridge, K. L., Munguia, J. E. & Waterman, M. L. *J. Biol. Chem.* **271**, 7654–7658 (1996).
- Cowin, P., Kapprell, J.-P., Franke, W. W., Tamkun, J. & Hynes, R. O. *Cell* **46**, 1063–1073 (1986).
- Giese, K., Cox, J. & Grosschedl, R. *Cell* **69**, 185–195 (1992).
- Love, J. J. et al. *Nature* **376**, 791–795 (1995).
- Kerppola, T. K. & Curran, T. *Cell* **66**, 317–326 (1991).
- Kratohvil, K., Dull, M., Farinas, I., Galceran, J. & Grosschedl, R. *Genes Dev.* **10**, 1382–1394 (1996).
- McMahon, A. P. & Moon, R. T. *Cell* **58**, 1075–1084 (1989).

- Sokol, S. Y., Klingensmith, J., Perrimon, N. & Itoh, K. *Development* **121**, 1637–1647 (1995).
- He, X., Saint-Jeannet, J.-P., Woodgett, J. R., Varmus, H. E. & Dawid, I. *Science* **374**, 617–622 (1995).
- Heasman, J. et al. *Cell* **79**, 791–803 (1994).
- Peifer, M., Sweeton, D., Casey, M. & Wieschaus, E. *Development* **120**, 369–380 (1994).
- Bhanot, P. et al. *Nature* **382**, 225–230 (1996).
- Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. & Polakis, P. *Proc. Natl. Acad. Sci. USA* **92**, 3046–3050 (1995).
- Miller, J. H. *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory Press, NY, 1972).
- Giese, K. & Grosschedl, R. *EMBO J.* **12**, 4667–4676 (1993).
- Carlson, P., Waterman, M. L. & Jones, K. A. *Genes Dev.* **7**, 2418–2430 (1993).
- Kim, J., Zwiebe, C., Wu, C. & Adhya, S. *Gene* **85**, 15–23 (1989).
- Krieg, P. & Melton, D. *Nucleic Acids Res.* **12**, 7057–7070 (1984).
- Kühl, M., Finnemann, S., Binder, O. & Wedlich, D. *Mech. Dev.* **54**, 71–82 (1996).

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CORRESPONDENCE and requests for materials should be addressed to W.B. (e-mail: wbirch@mdc-berlin.de).

An essential component of the decapping enzyme required for normal rates of mRNA turnover

Clare A. Beelman, Audrey Stevens*,
Giordano Caponigro, Thomas E. LaGrande†,
Lianna Hatfield‡, David M. Fortner & Roy Parker†

Department of Molecular and Cellular Biology, and †Howard Hughes Medical Institute, University of Arizona, Tucson, Arizona 85721, USA
*Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA

A MAJOR pathway of messenger RNA degradation in eukaryotic cells is initiated by shortening of the poly(A) tail, which, at least in yeast, triggers a decapping reaction, thereby exposing the mRNA to 5' → 3' degradation¹⁻⁴. Decapping is the key step in this decay pathway because the transcript body is rapidly degraded following decapping. Accordingly, decapping is the site of numerous controls, including inhibition of decapping by the poly(A) tail⁴ and modulation of mRNA decapping rate by specific sequences³⁻⁵. Moreover, a specialized decay pathway that degrades aberrant transcripts triggers rapid mRNA decapping independently of poly(A)-tail shortening⁶. We have identified a yeast gene, termed *DCPI*, that encodes the decapping enzyme, or an essential component of a decapping complex. The protein Dcp1 is required for the normal decay of many unstable and stable yeast mRNAs, as well as mRNAs that are decapped independently of deadenylation. These results indicate that mRNA-specific rates of decapping, and thus decay, will result from differences in

the interaction of the *DCPI* decapping enzyme with individual transcripts.

We have identified mutations in three genes that are required for normal rates of mRNA turnover⁷. Strains carrying one of these mutations, *dcp1-1* (see below), are unable to grow at 36 °C when combined with a second modifier mutation, *MDC1-1* (ref. 7). We identified a gene from a genomic yeast library that would complement this conditional growth phenotype (termed the *DCPI* gene) and suppress the mRNA decay defect in *dcp1-1* strains (data not shown). Two observations indicated that the *DCPI* gene is the site of the *dcp1-1* lesion. First, a *dcp1Δ* gene disruption was allelic to the *dcp1-1* mutation in twenty-one tetrads. Second, sequencing the *DCPI* gene from the *dcp1-1* strain identified a single point mutation, resulting in a glycine-to-aspartic acid substitution (Fig. 1a).

To determine the role of Dcp1 in mRNA decay *in vivo*, we analysed the effects of a *DCPI* gene disruption on mRNA turnover. Dissection of diploids heterozygous for a replacement of the *DCPI* coding region with the *URA3* gene (*dcp1Δ::URA3*) gave rise to *URA+* spores that grew slowly (doubling time of 265 min at 30 °C in rich medium) compared to wild-type cells (doubling time, 105 min). We examined the decay of the unstable MFA2pG transcript (the *MFA2* mRNA that contains a poly(G) tract in its 3' untranslated region, which allows detection of decay intermediates) in the *dcp1Δ* strain by a transcriptional pulse-chase experiment¹. In this experiment the *GAL1* promoter was used to induce, and then repress, transcription, thereby producing a 'pulse' of transcripts whose decay could be monitored during an ensuing 'chase'. A critical result was that *dcp1Δ* and *dcp1-1* cells showed essentially normal rates of deadenylation but accumulated full-length deadenylated transcripts as compared to a wild-type strain (Fig. 2a, and data not shown). Immunoprecipitation of transcripts from *dcp1Δ* or *dcp1-1* strains with antisera directed against the cap structure indicated that the full-length deadenylated transcripts that accumulated in these strains were capped (Fig. 2b, and data

‡Present address: Uintah Basin Campus, Utah State University, Vernal, Utah 84078, USA.

not shown). In contrast, an *xm1A* strain, which lacks the 5' to 3' exonuclease required to degrade mRNAs after decapping, accumulated deadenylated and decapped transcripts (Fig. 2b and refs 2, 3). These observations indicated that Dcp1 is required for decapping *in vivo*.

Several lines of evidence indicated that the *DCP1* gene encodes either the decapping enzyme, or an essential component of a decapping complex. In one case, the amino-terminal sequence of a polypeptide present in a highly purified preparation of decapping activity matched the predicted amino-acid sequence of Dcp1. This enzymatic activity, which releases m⁷GDP from the 5' end of transcripts⁸, was purified ~40,000-fold from yeast extracts (see Supplementary Information). Following initial steps in this purification, fractionation of the activity on a Mono-Q column yielded two peaks of activity (termed decap 1 and 2). Further purification of decap 1 and 2 on hydroxylapatite columns in each case identified a single, abundant polypeptide (20–50% of the total protein) that paralleled the enzymatic activity, and was approximately the size predicted for Dcp1 (*M*_r 28K for decap 1, and 30–31K for decap 2) (A.S., manuscript in preparation). Although the N-terminal sequence of the 28K protein was not obtained, N-terminal sequencing of the 30–31K protein from

decap 2 gave a 14-amino-acid sequence identical to the predicted Dcp1 amino-acid sequence (underlined in Fig. 1a).

Additional evidence that Dcp1 is a component of the decapping enzyme is that *dcp1Δ* strains showed essentially no decapping activity in cell-free extracts (Table 1a) and *dcp1-1* strains showed reduced activity (~15% of wild type; Table 1a). In addition, overexpression of the *DCP1* gene from either a multicopy plasmid or the *GAL1* promoter, substantially increased the amount of decapping activity (Table 1a). Overexpression of Dcp1 increased both decap 1 and decap 2 peaks (data not shown). This observation, and the lack of decapping activity in the *dcp1Δ* strain, suggested that decap 1 and decap 2 represent two different forms of Dcp1, possibly differing by post-translational modification or proteolysis.

A final line of evidence that Dcp1 is a component of the decapping enzyme came from analysis of a modified Dcp1, His-Dcp1, which contains a hexahistidine N-terminal extension capable of binding to nickel-affinity columns⁹. The His-Dcp1 complemented the mRNA turnover and slow growth defects due to *dcp1Δ* (data not shown). Importantly, the majority of decapping activity (Fig. 1b) and His-Dcp1 (Fig. 1c) expressed in the *dcp1Δ* strain copurified on a nickel-affinity column. In contrast, decap-

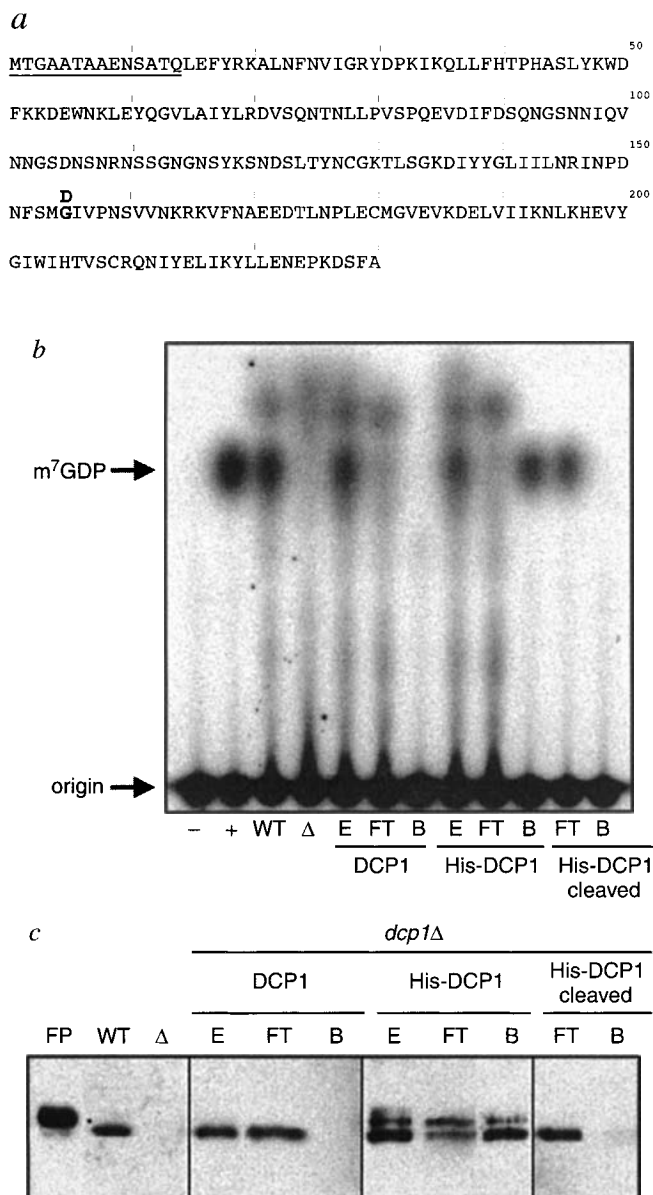


FIG. 1a, Predicted amino-acid sequence of Dcp1. The N-terminal sequence obtained from the purified decap 2 polypeptide is underlined. The amino-acid substitution in the *dcp1-1* allele is shown in bold and results from a GGC to GAC change at codon 155. b, PEI-cellulose TLC separation of *in vitro* decapping reactions. Lanes are as follows: -, substrate alone; +, purified decap 2; WT, wild-type (yRP840) whole-cell extract; Δ, *dcp1Δ* (yRP1070) whole-cell extract; DCP1 lanes are samples from yRP1070 expressing the DCP1 gene from pRP776; His-Dcp1 lanes are samples from yRP1070 expressing the His-Dcp1 gene from pRP777; His-Dcp1 (cleaved) lanes show the analysis of purified His-Dcp1 after proteolytic cleavage of the His tag from Dcp1; E, whole-cell extract; FT, flow-through from the nickel-NTA histidine affinity column; B, imidazole eluate. c, Western blot analysis of crude extracts and column fractions using Dcp1-specific antibodies. FP, His-Dcp1 expressed in *E. coli*. Other lanes correspond to samples used for PEI-cellulose TLC analysis as listed in b.

METHODS. a, The *DCP1* gene was identified on an ~9-kb fragment from a genomic library that complemented the *dcp1-1*, *MDC1-1* temperature-sensitive phenotype (Genbank accession number, Z48239; *DCP1* corresponds to the third ORF in this sequence). Subcloning the *Nsi*-*Cla*I fragment, containing the third ORF of this region, into pRS416, and pRS424 yielded plasmids pRP717(CEN) and pRP718(2 μM), which complemented the *dcp1-1*, *MDC1-1* phenotypes. The *DCP1* gene disruption replaced the *DCP1* coding region from the 5' *Eco*RI site to the 3' *Dra*I site with the *URA3* gene (pRP716). The *dcp1-1* allele was sequenced following PCR amplification. b, Cells were collected after growth in selective media, washed with lysis buffer (10 mM Tris-HCl, pH 7.6, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT), and suspended in an equal volume of lysis buffer containing 2 μg ml⁻¹ each of leupeptin and pepstatin A. Acid-washed glass beads equal in volume to the cell suspension were added, and cells were lysed by six cycles of vortexing for 15 s and 45 s of cooling in ice water. The lysate was centrifuged at 12,000g for 15 min at 4 °C, and the supernatant chromatographed by gravity flow on a 1 ml Nickel-NTA column (Qiagen/GibcoBRL) and eluted stepwise with 2-ml aliquots of buffer C (20 mM Tris-HCl, pH 8.5, 0.1 M KCl, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol) containing increasing concentrations of imidazole (0.1, 0.2, 0.3, 0.4 or 0.5 M); 1-ml fractions were collected. Most His-Dcp1 eluted at 0.2 M imidazole; the His tag was removed by cleavage with TEV protease (GibcoBRL). Column fractions were assayed for decapping activity at 30 °C for 10 min in reactions containing ~0.02 pmol of m⁷G[³²P]pppMFA2 RNA, 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 50 mM NH₄Cl, and 0.4 mM DTT, and analysed by PEI-cellulose TLC developed in 0.45 M (NH₄)₂SO₄. The substrate MFA2 mRNA was synthesized *in vitro* with T7 RNA polymerase from a PCR-amplified DNA template²⁰, and the RNA capped at 37 °C for 2 h in a reaction containing 23 pmol RNA, 45 pmol [α-³²P]GTP, 40 U RNasin (Promega), 0.67 mM S-adenosylmethionine, 50 mM Tris-HCl, pH 7.6, 2 mM MgCl₂, 1 mM DTT and 25 U guanylyltransferase (GibcoBRL). Cap-labelled RNAs were separated from unincorporated label by Sepharose-CL6B (Pharmacia) spin chromatography. For c, Standard protocols were followed for western analysis and ECL chemiluminescence (Amersham) using affinity-purified Dcp1-specific antisera.

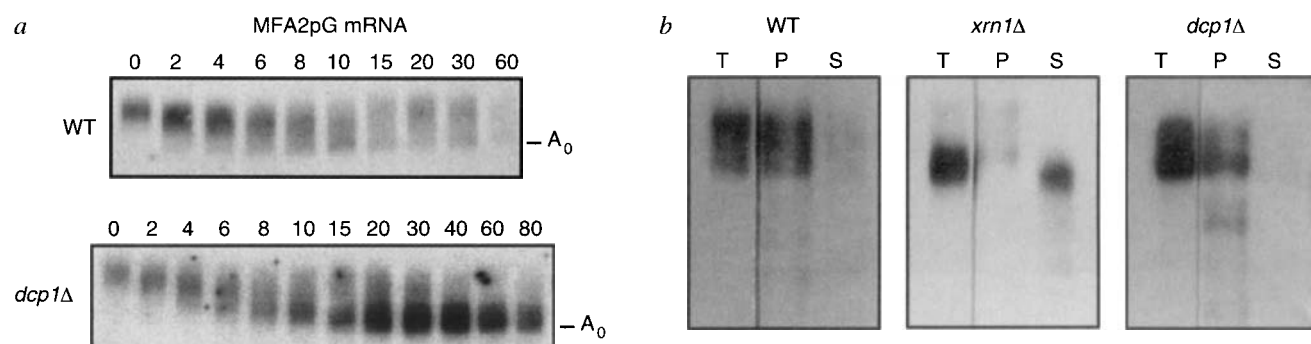


FIG. 2 Deletion of the *DCP1* gene inhibits decapping of the MFA2pG transcript. *a*, Polyacrylamide northern blots probed for the MFA2pG RNA across a transcriptional pulse-chase. Transcription was induced for 8 min, followed by repression of transcription at time zero by the addition of glucose. Time points represent minutes after transcriptional repression. Strains used were wild type (yRP840) and a congenic *dcp1Δ* strain (yRP1069) (*MATa*, *trp1*, *ura3-52*, *leu2-3,112*, *his4-539*, *cup1::LEU2* MFA2pG PGK1pG, *dcp1::URA3*). The appearance of fully adenylated mRNA seen in both the wild type and *dcp1Δ* strains at 15–30 min is due to some residual transcription. *b*, Immunoprecipitations of steady-state

MFA2pG transcripts, using antisera directed against the 5'-cap structure, from wild-type, *dcp1Δ*, and *xrn1Δ* strains grown in medium with galactose as the carbon source. In each case, transcripts were analysed on polyacrylamide northern gels, where both *dcp1Δ* (yRP1069) and *xrn1Δ* (yRP1062) strains accumulated deadenylated mRNAs. Lanes are labelled as follows: T, total input RNA; P, immunoprecipitate pellet; S, supernatant. The total lanes have been underexposed to allow the distribution of transcript present at steady state to be observed; this was necessary because some of the input RNA is lost during immunoprecipitation. METHODS. Experiments followed previously described protocols^{1,3,21}.

ping activity and wild-type Dcp1 expressed in the *dcp1Δ* strain did not bind to a nickel-affinity column (Fig. 1*b, c*). Moreover, after proteolytic cleavage of the hexahistidine extension from His-Dcp1, the decapping activity and Dcp1 did not bind to a nickel-affinity column (Fig. 1*b, c*). Interestingly, in *dcp1Δ* strains expressing His-Dcp1, two proteins were detected on western blots (Fig. 1*c*). This observation is similar to the detection of two separable forms of Dcp1 in wild-type yeast (decap 1 and decap 2), and is consistent with the hypothesis that Dcp1 may undergo post-translational modification or proteolysis.

We interpreted the biochemical evidence (Table 1*a* and Fig. 1*b*), and the strong block to decapping seen *in vivo* in *dcp1* mutant strains, to indicate that the *DCP1* gene encodes the decapping enzyme, or an essential component of the decapping enzyme. Importantly, as Dcp1 promotes the release of m⁷GDP from transcripts *in vitro*, and is required for mRNA decapping *in vivo*, this suggests that decapping of mRNAs *in vivo* is characterized by removal of m⁷GDP from transcripts.

As mRNA deadenylation leading to decapping has been predicted to be a common pathway of mRNA degradation^{3,10,11}, we

TABLE 1 mRNA decapping activity and half-lives in yeast strains

(a) mRNA decapping activity				(b) mRNA half-lives in wild-type and <i>dcp1Δ</i> yeast strains		
Expt	Strain	Plasmid	Relative activity	mRNA	Wild type	<i>dcp1Δ</i>
(1)	Wild type	None	1.0*	MFA2pG	6'	20'
	<i>dcp1Δ</i>	None	<0.05*	GAL10	5'	20'
(2)	Wild type	None	1.0†	HIS3	7'	18'
	<i>dcp1-1</i>	None	0.15†	PGK1pG	25'	45'
	<i>dcp1Δ</i>	None	<0.05†	ACT1	17.5'	32'
	<i>dcp1Δ</i>	None	<0.05†	pre-CYH2	2'	23'
(3)	Wild type	p424 (2 μM)	1.0†	CYH2	26'	>45'
	Wild type	p424-DCP1 (2 μM)	5.4†			
(4)	Wild type -GAL	pGAL-DCP1	1.0†			
	Wild type +GAL	pGAL-DCP1	9.0†			

For *a*, mRNA decapping enzyme activity was measured using ³H-cap-labelled TAT mRNA as described⁸. Activity was calculated as units per mg protein and is expressed as relative activity. Yeast cells were grown in YEPD medium (expts 1 and 2) or supplemented SD medium (expt 3) to late log phase. Cells in experiment 3 were grown as described¹⁸. Yeast cells (25 g) were disrupted in a Beadbeater and the suspension was centrifuged and processed (see Supplementary Information) to obtain the washed 27,000g pellet. The washed pellet was suspended by homogenization in 8 ml high-salt buffer, stirred for 1 h, then centrifuged for 135 min at 45,000 r.p.m. in a Spinco 50 Ti rotor and the supernatant removed. This high-salt-wash fraction, which contains 80–90% of the mRNA decapping activity, was diluted with an equal volume of buffer A (20 mM Tris-HCl buffer pH 7.6, 10% glycerol, 0.5 mM DTT, 0.1% Triton X-100) and applied to a heparin-agarose column (5 ml) prewashed with buffer A containing 250 mM (NH₄)₂SO₄. The column was then washed with 7.5 ml of the same buffer. For *b*, half-lives of MFA2pG, PGK1pG and GAL10 mRNAs were determined in yRP840 (ref. 14) and a congenic *dcp1Δ* strain (yP1069) following glucose repression of transcription as described. Half-lives of the *HIS3*, *CYH2* and *ACT1* mRNAs were determined in strain yRP1106 (*MATa*, *dcp1::URA3*, *rbp1-1*, *ura3-52*, *leu2-3,112*), and a congenic *DCP1* strain following repression of RNA polymerase II (as described in ref. 10). The PGK1pG and *ACT1* transcripts were less stable in the wild-type strain than previously reported (see ref. 10 for example) because of the different strain background. mRNA was quantified with a phosphorimager (Molecular Dynamics) following hybridizing of northern blots with either 5' ³²[P]-end-labelled oligonucleotides specific for the MFA2pG (oRP140; ref. 14), PGK1pG (oRP141; ref. 14), GAL10 (oRP98; ref. 1) and *ACT1* (oRP166; ref. 19) transcripts, or random-primed DNA probes specific for the *HIS3*, *CYH2* and mRNAs.

* For these assays, a gradient elution was used and the activity peak analysed.

† For these assays, the decapping enzyme was eluted with buffer A containing 700 mM (NH₄)₂SO₄.

examined the decay of several transcripts in the *dcp1Δ* strain. Two observations suggested that Dcp1 was required for normal decay of the stable *PGK1* transcript, which in this case contained a poly(G) insertion in the 3' UTR. First, the *PGK1pG* transcript was more stable in *dcp1Δ* strains as compared to congenic wild-type strains (Table 1b; Fig. 3a). This was due to a slower rate of decay following deadenylation, consistent with a block to decapping (data not shown). Second, an intermediate arising by 5' to 3' degradation to the poly(G) tract¹ was lacking in *dcp1Δ* cells while levels of intermediates produced by an alternative 3' to 5' decay pathway⁴ were increased (Fig. 3b). Measurement of decay rates for a number of additional mRNAs indicated that the *dcp1Δ* mutation slows the turnover of several yeast mRNAs, including transcripts with rapid, intermediate and slow decay rates (Table 1b).

Transcripts that contain early non-sense codons are degraded through a deadenylation-independent decapping reaction⁶. This is part of a mRNA surveillance pathway for degrading aberrant transcripts (see refs 10, 12). In *dcp1Δ* and *dcp1-1* strains, the rapid decay of a transcript containing an early nonsense codon (^{B55}*PGK1*^{N103}pG) and the decay of the unspliced *CYH2* precursor mRNA, which is thought to be degraded by the same mechanism¹³, was inhibited (Fig. 3c, Table 1b, and data not shown). This result

indicated that Dcp1 decapping activity is required for deadenylation-independent decapping.

These results show that a single decapping enzyme is required for the decapping of many unstable and stable yeast mRNAs, as well as mRNAs that are decapped independently of deadenylation. This observation implies that features of mRNAs that specify different rates of decapping do so by affecting the cleavage of individual transcripts by Dcp1-dependent decapping activity. The current evidence indicates that Dcp1 activity on transcripts may be modulated by proteins that recognize specific mRNA features. For example, inhibition of decapping by the poly(A) tail requires the poly(A)-binding protein¹⁴. Similarly, the rapid deadenylation-independent decapping triggered by premature translational termination requires the products of the *UPF1*,

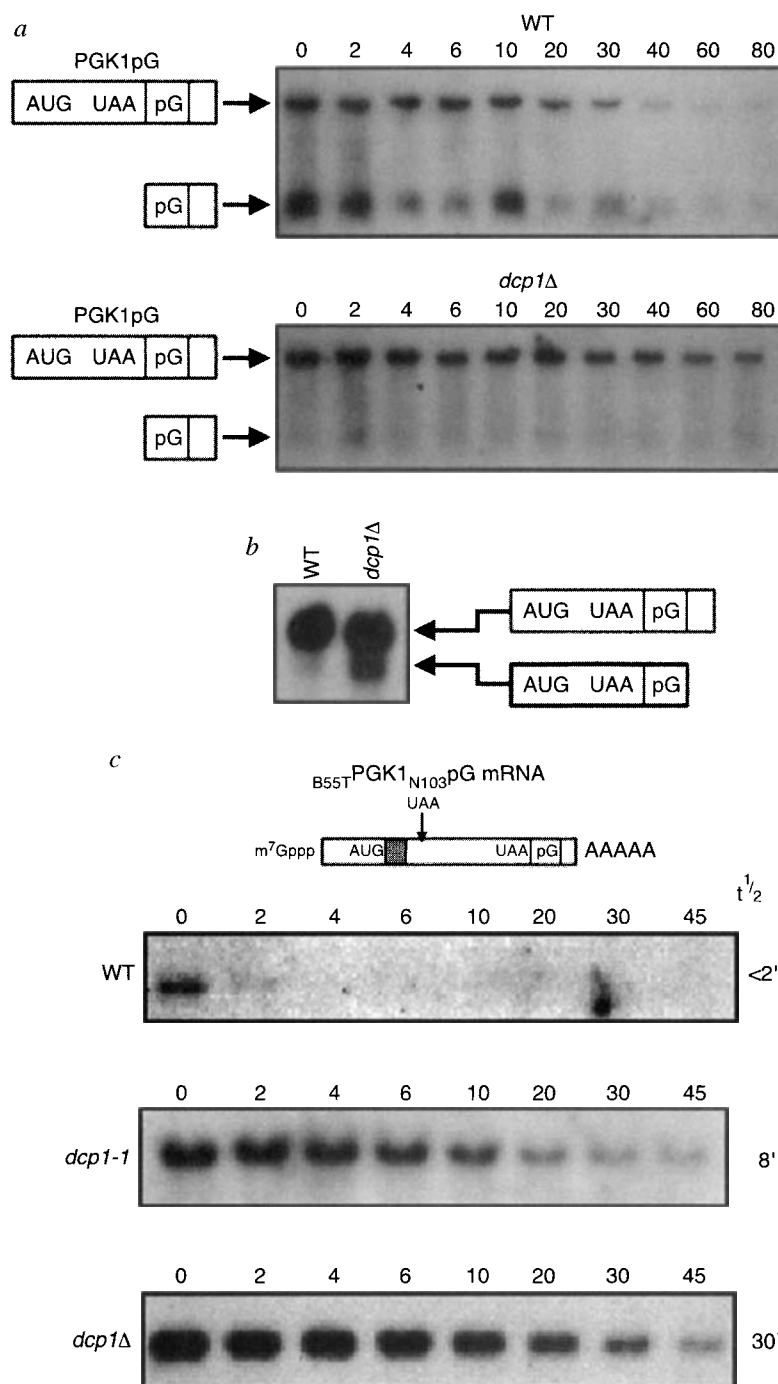


FIG. 3 Dcp1 is required for decay of *PGK1* transcripts. **a**, Analysis of the *PGK1pG* transcript in wild-type (top panel) and *dcp1Δ* (bottom) strains on agarose northern blots. Blots were probed with an oligonucleotide (oRP141; ref. 14) that hybridizes to full-length transcripts and mRNA decay products arising from 5' to 3' decay³. The 10-min timepoint in the wild-type timecourse is slightly overloaded, as judged by RNA standardization to 7S RNA. **b**, Comparison of *PGK1pG* transcripts in wild-type and *dcp1Δ* strains on a northern gel using an oligonucleotide probe (oRP154; ref. 6) that allows detection of full-length transcripts and mRNA decay products arising from 3' to 5' decay⁴. **c**, Decay of the ^{B55}*PGK1*^{N103}pG transcript⁶, which contains an early nonsense codon, in wild-type (yRP840), *dcp1Δ* (yRP1069), and *dcp1-1* (yRP891) strains following transcriptional repression. A plasmid expressing the ^{B55}*PGK1*^{N103}pG transcript was introduced into these strains and was maintained by growing the strains in selective medium containing galactose to induce transcription of the ^{B55}*PGK1*^{N103}pG transcript. Transcription was shut-off at time zero by resuspending cells in selective medium containing glucose. To prevent cross-hybridization to the chromosomally expressed *PGK1* and *PGK1pG* transcripts, the northern blots were probed with an oligonucleotide specific for the ^{B55}*PGK1*^{N103}pG transcript (oRP252: 5'-CTTGA-CAGAGATCAATTCG-3').

METHODS. For **a** and **b**, wild-type (yRP840) and an congenic *dcp1Δ* strain (yRP1069) were grown in YEP medium containing 2% galactose and 2% sucrose as carbon sources. Transcription was repressed by the addition of glucose to 2% and time points were taken as described⁴.

UPF2 and *UPF3* genes^{13,15-17}. Finally, the *MRT1* and *MRT3* gene products, which are only required for deadenylation-dependent decapping⁷, appear to promote decapping following deadenylation. These proteins may all directly affect the interaction of the decapping enzyme with each mRNA. Alternatively, modulation of decapping rate may occur by alterations in mRNP structure that affect the accessibility of the cap structure to the decapping enzyme. For example, control of decapping rate may occur through competition for the 5' cap structure between the eIF-4F cap binding complex and the decapping enzyme. Such a model is appealing in that it would provide a mechanistic basis for the observation that the translation and turnover of eukaryotic mRNAs are often coupled. □

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1. Decker, C. J. & Parker, R. *Genes Dev.* **7**, 1632-1643 (1993).
2. Hsu, C. L. & Stevens, A. *Molec. cell. Biol.* **13**, 4826-4835 (1993).
3. Muhlad, D., Decker, C. J. & Parker, R. *Genes Dev.* **8**, 855-866 (1994).
4. Muhlad, D., Decker, C. J. & Parker, R. *Molec. cell. Biol.* **15**, 2145-2156 (1995).
5. Muhlad, D. & Parker, R. *Genes Dev.* **6**, 2100-2111 (1992).
6. Muhlad, D. & Parker, R. *Nature* **370**, 578-581 (1994).
7. Hatfield, L., Beelman, C. A., Stevens, A. & Parker, R. *Molec. cell. Biol.* (in the press).

8. Stevens, A. *Molec. cell. Biol.* **8**, 2005-2010 (1988).
9. Hochuli, E., Döbeli, H. & Schacher, A. *J. Chromatogr.* **411**, 177-184 (1987).
10. Beelman, C. A. & Parker, R. *Cell* **81**, 179-183 (1995).
11. Decker, C. J. & Parker, R. *Trends biochem. Sci.* **19**, 336-340 (1994).
12. Peltz, S. W., He, F., Welch, E. & Jacobson, A. *Prog. Nucleic Acids Res. molec. Biol.* **17**, 271-298 (1994).
13. He, F. & Jacobson, A. *Genes Dev.* **9**, 437-454 (1995).
14. Caponigro, G. & Parker, R. *Genes Dev.* **9**, 2421-2432 (1995).
15. Leeds, P., Peltz, S. W., Jacobson, A. & Culbertson, M. R. *Genes Dev.* **5**, 2303-2314 (1991).
16. Leeds, P., Wood, J. M., Lee, B.-S. & Culbertson, M. R. *Molec. cell. Biol.* **12**, 2165-2177 (1992).
17. Cui, Y., Hagan, K. W., Zhang, S. & Peltz, S. W. *Genes Dev.* **9**, 423-436 (1995).
18. Johnson, A. W. & Kolodner, R. D. *J. biol. Chem.* **266**, 14046-14054 (1991).
19. Mandart, E. & Parker, R. *Molec. cell. Biol.* **15**, 6979-6986 (1995).
20. Harris, M. E. et al. *EMBO J.* **13**, 3953-3963 (1994).
21. Caponigro, G., Muhlad, D. & Parker, R. *Molec. cell. Biol.* **13**, 5141-5148 (1993).

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CORRESPONDENCE and requests for materials should be addressed to R.P. (e-mail: parker_lab@tikal.biosci.arizona.edu).

Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide

Maria J. Macias, Marko Hyvönen, Elena Baraldi, Johan Schultz*, Marius Sudol†, Matti Saraste & Hartmut Oschkinat

European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

* Forschungsinstitut für Molekulare Pharmakologie, Alfred-Kowalke-Str. 4, 10315 Berlin, Germany

† Mount Sinai School of Medicine, Department of Biochemistry, Box 1020, One Gustave L. Levy Place, New York, New York 10029-6574, USA

THE WW domain is a new protein module with two highly conserved tryptophans that binds proline-rich peptide motifs *in vitro*. It is present in a number of signalling and regulatory proteins, often in several copies¹⁻³. Here we investigate the solution structure of the WW domain of human YAP65 (for Yes kinase-associated protein) in complex with proline-rich peptides containing the core motif PPxY (ref. 4). The structure of the domain with the bound peptide GTPPPYTVG is a slightly curved, three-stranded, antiparallel β -sheet. Two prolines pack against the first tryptophan, forming a hydrophobic buckle on the convex side of the sheet. The concave side has three exposed hydrophobic residues (tyrosine, tryptophan and leucine) which form the binding site for the ligand. A non-conserved isoleucine in the amino-terminal flanking region covers a hydrophobic patch and stabilizes the WW domain of human YAP65 *in vitro*. The structure of the WW domain differs from that of the SH3 domain and reveals a new design for a protein module that uses stacked aromatic surface residues to arrange a binding site for proline-rich peptides.

The WW domain forms a new family of protein modules¹⁻³, analogous to the Src-homology-2 and -3 (SH2, SH3), pleckstrin-homology (PH) and phosphotyrosine-binding (PTB) domains⁵⁻⁹. It consists of ~38 amino acids¹ with a high content of hydrophobic, aromatic and proline residues (Fig. 1), and two highly conserved tryptophans (hence WW domain). Like other intracellular protein modules, the WW domain is present in a variety of cyto-

skeletal and signal-transducing proteins, such as YAP65, dystrophin, ubiquitin-protein ligases, the transcriptional activator FE65 and proteins binding to formin^{1-3,10}. It binds proline-rich peptides, as do SH3 domains^{11,12}, and WW and SH3 domains may compete for binding to the same sequence motifs¹⁰. The WW domain of human YAP (hYAP) binds peptides with a consensus sequence PPxY *in vitro*⁴. A WW domain is necessary for control of epithelial sodium channels: carboxy-terminal deletions of the amiloride-sensitive channel cause Liddle's syndrome, a hereditary form of hypertension. The C terminus contains PPxY motifs that interact with a WW domain in the regulatory protein Nedd4 (refs 13, 14), indicating that WW domains may be alternative recognition sites for proline-rich ligands.

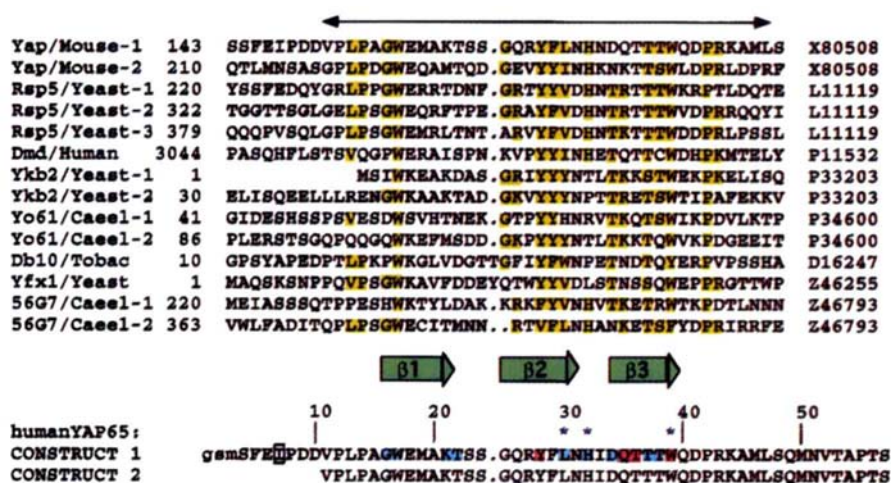
Several constructs of the WW domain hYAP with variable N- and C-terminal extensions were made and expressed in *Escherichia coli*. Although intact fusion proteins with glutathione-S-transferase (GST) were always obtained, only two could be purified after cleavage with thrombin (Fig. 1, bottom). Construct 1 was folded, as indicated by a number of amide proton signals between 8.5 and 9.5 p.p.m. in the ¹H NMR spectrum. From the multiple sequence alignment (Fig. 1), construct 2 should already contain the full-length domain, but its NMR spectrum did not show any amide resonances with chemical shifts larger than 8.5 p.p.m., the signals of the aromatic residues were not dispersed, and the two-dimensional spectra gave a peak pattern typical of a random coil. We therefore used construct 1 in all further experiments, which was also labelled with ¹³C and ¹⁵N.

We determined the structure of the hYAP WW domain in a complex with the proline-rich peptide GTPPPYTVG. The domain structure consists of a twisted and slightly bent three-stranded antiparallel β -sheet (Fig. 2a) comprising residues 16-22, 26-32 and 35-39. It is well ordered in the region L13-P42 (Fig. 2b and Table 1), where residues 14-17, 23-25, 33-34 and 40-42 form well defined turns. Residues Y28 and W39 are located on the bottom side, and F29 and W17 are on the top side of the sheet (Fig. 2b). The side chains of Y28 and W39 fill its concave side so that an almost flat hydrophobic surface is produced. The N and C termini of the domain meet on the convex side to form a hydrophobic buckle, in which the side chains of P14 and P42 are positioned opposite each other above W17.

The stretches comprising residues 1-6, 8-12, and 43-50 show only few weak long- or medium-range nuclear Overhauser effects (NOEs) (11 → 43, 43 → 45) and appear to be flexible. The core domain as defined by the sequence alignment has an exposed hydrophobic surface in the front of the buckle. This is covered by isoleucine at position 7 (red in Fig. 2b) like a snap fastener, with

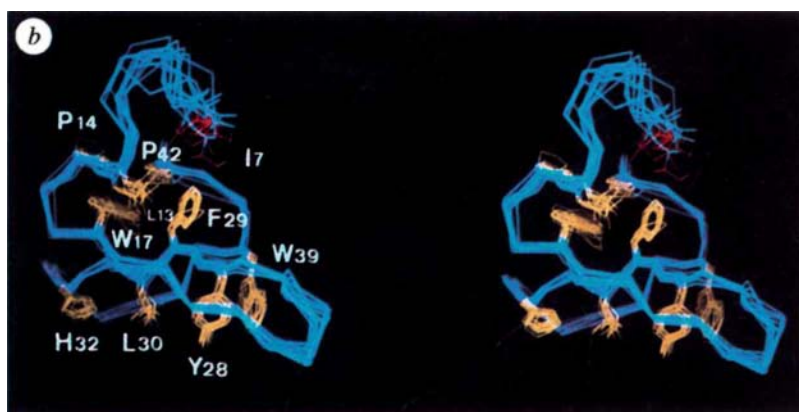
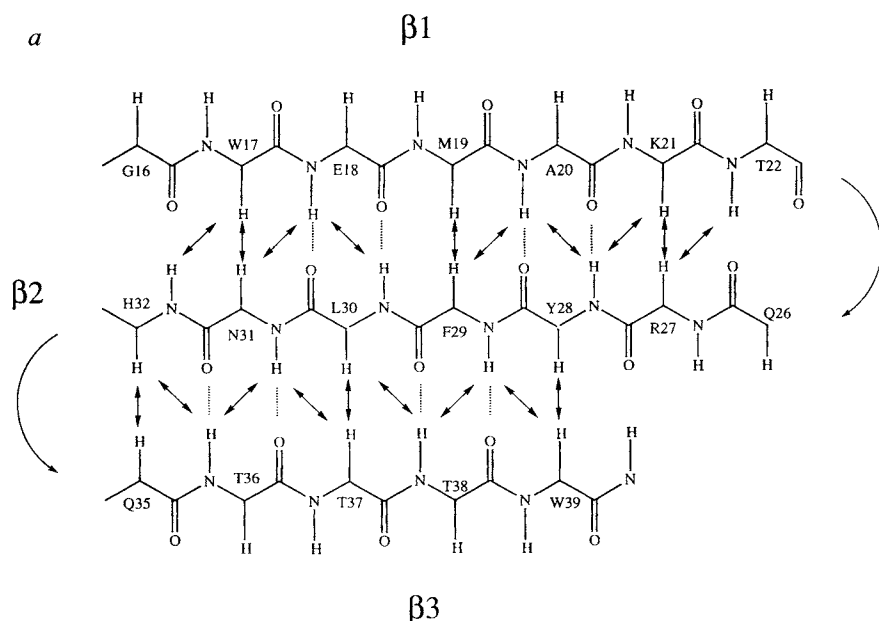
FIG. 1 Sequence alignment of WW domains from selected proteins. Residue numbers are shown on the left, and accession numbers on the right. Bottom, constructs 1 and 2 of the WW domain from human YAP65; residue numbering is based on construct 1. Residues showing strong chemical shift changes upon peptide binding (>0.5 p.p.m.) are indicated in red; those showing smaller changes (0.2 p.p.m.) are labelled in blue. Residues with a star above the sequence of construct 1 show NOEs to the ligand peptide. The isoleucine residue at position 7 that is essential for folding is in an open box. The domain boundaries originally proposed¹ are indicated by an arrow.

METHODS. Both constructs were amplified by using the polymerase chain reaction (PCR) and cloned human YAP65 cDNA as template. Amplified fragments were cloned into GST fusion vector pGAT2 (J. Peränen and M.H., unpublished). The expressed fusion protein contains both a His-tag and GST, allowing double-affinity purification. The WW domain was released from the fusion protein by thrombin. All DNA constructs were verified by dideoxy sequencing. Proteins were expressed in the *E. coli* strain BL21(DE3) and purified by affinity chromatography on glutathione-Sepharose (Pharmacia), Ni^{2+} -NTA-agarose (QIAGEN), and gel



filtration on a Superdex-30 (Pharmacia) column (details of purification available on request). Relative molecular masses of purified proteins were determined by electrospray mass spectrometry. The three mutants were made by PCR mutagenesis using construct 1 as a template; they were cloned and expressed as the wild-type protein.

FIG. 2 a, Secondary structure NOE pattern of the WW domain. Hydrogen bonds are indicated by dotted lines if the predicted inter-strand NOEs are present. Series of COSY, NOESY and clean TOCSY¹⁸⁻²⁰ spectra of an unlabelled construct 1 with and without the peptide ligand (protein concentration 1.2 mM, peptide:protein ratio 3:1, 10 mM potassium phosphate, pH 6, 100 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA, 0.02% NaN_3) were recorded at 600 MHz and 285 K and 278 K, leading to nearly complete assignments. HCCH-TOCSY²¹ and 3D ^{13}C -resolved NOESY^{22,23} spectra were measured using [^{13}C , ^{15}N]-labelled protein and unlabelled peptide. Various $^{13}\text{C}/^{12}\text{C}$ -filtered spectra²⁴ were used to differentiate between intermolecular and intramolecular NOEs and to assign signals from the bound peptide. Owing to the broadness of the signals in the environments of the bound peptide and P42 (residue numbering refers to construct 1; Fig. 1), a NOESY spectrum was recorded at 750 MHz in D_2O . This yielded sharper lines and therefore additional NOEs, especially between P14 and P42, and between the WW domain and the bound peptide. b, Superposition of 13 structures with the lowest energy, showing the backbone of residues 17–P42 and the side chains of I7, P14, W17, Y28, F29, L30, H32, I33, W39 and P42. The side chain of I7 that is necessary for the folding of the domain is shown red, all others are yellow. The structures were calculated using a standard simulated annealing protocol¹⁵ and X-PLOR¹⁶. For structure statistics, see Table 1.



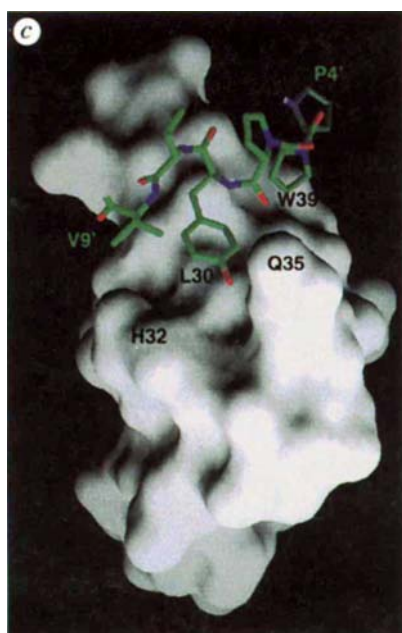
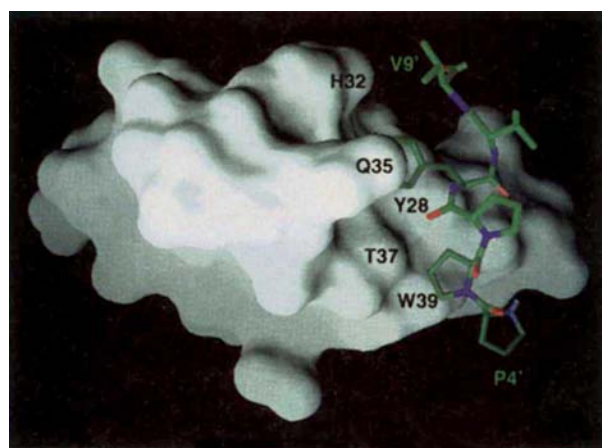
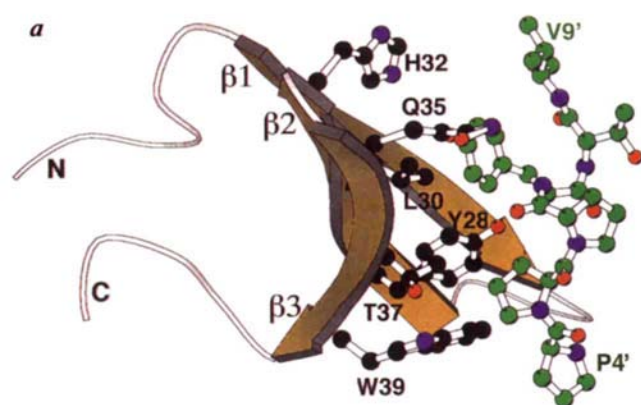


FIG. 3 a, A model of the peptide/WW-domain complex drawn with MOLSCRIPT²⁵. Only the residues of the peptide showing contacts to the protein are shown (P4'–V9'). The peptide was modelled by using ten intermolecular NOEs and restraining the conformation of the proline segment into a polyproline type II polyproline. The side chains of the domain (carbon atoms in black) and ligand (in green) residues that are involved in the interaction are shown. Nitrogen atoms are blue, oxygen atoms red. b, c, The model of the complex drawn with GRASP²⁶ shows the van der Waals surface of the WW domain and the bound peptide ligand as a stick presentation.

contacts to F29, P14 and P42. This residue is outside the domain core and may be uniquely important for the stability of the hYAP WW domain as it is not conserved in the N-terminal flanking region (Fig. 1).

As the consensus of the hYAP WW ligand is PPxY (ref. 4), we used two peptides for structural studies (GTPPPPYTVG and SPPPPYTV) that had this sequence at residues 4'–7' and 2'–5', respectively (peptide residues are primed). The binding site for both peptides, as indicated by chemical shift changes (Fig. 1) and peptide-protein NOEs, corresponds to the area that is defined by residues Y28, L30, H32 and six residues from D34 to W39 on the concave side of the domain. This area presents a large hydrophobic patch on the protein surface formed by the side chains of Y28, L30, W39 and a number of threonine methyl groups. The pattern of intermolecular NOEs is formed by contacts between the ligand residues P5' and W39, Y7' and L30 and H32, and V9'

TABLE 1 Structure statistics and binding constants of complexes

Structure statistics		
Number of constraints		
sequential		160
medium-range		87
long-range		219
intramolecular		30
hydrogen-bond mimics (2 NOEs each)		8
intermolecular		10
Number of NOEs per residue (13–43)		
		15
X-PLOR potential energy (kcal m ⁻¹)		
E_{total}		184 ± 5
E_{bonds}		13 ± 1
E_{angles}		92 ± 4
$E_{\text{impropers}}$		7 ± 2
E_{vdW}		17 ± 3
E_{NOE}		44 ± 2
R.m.s.d. from ideal values		
angles		0.60 ± 0.02
impropers		0.41 ± 0.02
R.m.s.d. (Å) between average (SA) and selected set of structures (SA) for residues 13–43		
	Backbone	All heavy
(SA) versus (SA)	0.54 ± 0.20	0.90 ± 0.20
Binding constants of mutant proteins and peptides		
Protein	Peptide	Binding constant (μM)
WT	SPPPPYTV	47 ± 6
WT	GTPPPPYTVG	52 ± 6
L30K	SPPPPYTV	n.b.
L30K	GTPPPPYTVG	40 ± 5*
H32A	SPPPPYTV	n.b.
H32A	GTPPPPYTVG	n.b.
Q35A	SPPPPYTV	n.b.
Q35A	GTPPPPYTVG	160 ± 18
WT	SPPPPATV	n.b.
WT	SPPPPPLTV	n.b.
WT	SPPPPFTV	118 ± 17
WT	SAPPPYTV	53 ± 17
WT	SPAPPYTV	n.b.
WT	SPPAPYTV	n.b.
WT	SPPPAYTV	63 ± 17

SA, simulated annealing; WT, wild type; n.b., no binding observed by fluorescence spectroscopy. Dissociation constants were determined by changes in the fluorescence emission spectra of our WW construct 1 upon addition of peptide at defined concentrations; calculations were made assuming formation of a 1:1 complex¹⁷. Fluorescence was measured at 25 °C in an Aminco Bowman series-2 luminescence spectrophotometer. Excitation wavelength was 298 nm, using a slit width of 2 nm. Fluorescence spectra were recorded from 310 to 360 nm wavelength, using a slit width of 4 nm. The protein concentration was kept at 10 μM in a 40 mM phosphate buffer at pH 7.2.

* This value does not agree with the other data and must be due to a structural rearrangement.

and H32. The NOEs clearly define the orientation of the ligand peptide on the WW domain.

The proline-containing part of peptide GTPPPPYTVG was restrained into a polyproline helix type II, and the structure of the complex was calculated using a standard simulated annealing protocol¹⁵ (X-PLOR¹⁶) and the ten intermolecular NOEs as constraints. The result shows smooth contacts between the ligand and the domain (Fig. 3). The central prolines P4' and P5' contact W39, and the carbonyl group of P6' points towards the OH group of the conserved residue Y28. The peptide tyrosyl residue Y7' is accommodated in a hydrophobic pocket formed by L30 and H32. These contacts are well defined by six NOEs between the aromatic ring of Y7' and the side chains of L30 and H32. Y7' could form a hydrogen bond to the histidine ring but also to Q35, whose chemical shifts change strongly on peptide binding (Fig. 1).

The aromatic residues at positions 39 and 28, a hydrophobic residue at position 30 and a histidine at position 32 all tend to be conserved (Fig. 1). Our structure shows that these are the residues that are in contact with the peptide. The importance of this hydrophobic surface is underscored by the low binding affinity of mutants H32A, L30K and Q35A to both peptides (except in one case, see Table 1; E.B. *et al.*, unpublished results). The structure of

the mutants remained intact, as judged by their two-dimensional NMR spectra. The binding affinities of peptides in which the tyrosine residue of SPPPPYTV is substituted with alanine, leucine or phenylalanine show that the YAP domain is specific for a tyrosine-containing motif. In the first two cases there is no binding, and the peptide containing phenylalanine has only a weak affinity (Table 1). Thus, the tyrosine in the PPxY motif may be needed for the interaction of ligands with a set of WW domains. The importance of the polyproline motif is shown by the lack of binding when the two centre prolines in this peptide are replaced by alanines, whereas replacement of the first proline has no effect (Table 1).

Our structure confirms the hypothesis⁴ that the WW domain is a binding module for proline-rich ligands. The PPxY motif may not be the only ligand for WW domains: for instance, it is not present in the proline-rich tail of formin that interacts with SH3 and WW domains¹⁰, where sequence motifs such as PPxLP are found. The structure indicates that hydrophobic residues replacing tyrosine of the ligand could be accommodated on the surface of those domains that contain hydrophobic residues other than leucine at position 30.

Note added in proof: An involvement of hYAP in retroviral budding through its WW domain was recently suggested²⁷. □

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1. Bork, P. & Sudol, M. *Trends biochem. Sci.* **19**, 531–533 (1994).
2. Andre, B. & Springael, J. Y. *Biochem. biophys. Res. Commun.* **205**, 1201–1205 (1994).
3. Hofmann, K. & Bucher, P. *FEBS Lett.* **358**, 153–157 (1995).
4. Chen, H. I. & Sudol, M. *Proc. natn. Acad. Sci. U.S.A.* **92**, 7819–7823 (1995).
5. Pawson, T. *Nature* **373**, 573–580 (1995).
6. Cohen, G. B., Ren, R. & Baltimore, D. *Cell* **80**, 237–248 (1995).
7. Sudol, M., Chen, H. I., Bougeret, C., Einbond, A. & Bork, P. *FEBS Lett.* **369**, 67–71 (1995).
8. Bork, P. & Margolis, B. *Cell* **80**, 693–694 (1995).
9. Zhou, M. M. *et al. Nature* **378**, 587–592 (1995).
10. Chan, D. C., Bedford, M. T. & Leder, P. *EMBO J.* **15**, 1045–1054 (1996).
11. Cicchetti, P., Mayer, B. J., Thiel, G. & Baltimore, D. *Science* **257**, 803–806 (1992).
12. Ren, R., Mayer, B. J., Cicchetti, P. & Baltimore, D. *Science* **259**, 1157–1161 (1993).
13. Staub, O. *et al. EMBO J.* **15**, 2371–2380 (1996).
14. Schild, L. *et al. EMBO J.* **15**, 2381–2387 (1996).
15. Nilges, M., Clore, G. M. & Gronenborn, A. M. *FEBS Lett.* **229**, 317–324 (1988).
16. Brünger, A. T. *X-PLOR 3.1 Manual* (Yale Univ. Press, New Haven, 1992).
17. Viguera, A. R., Arrondo, J. L. R., Musacchio, A., Saraste, M. & Serrano, L. *Biochemistry* **133**, 10925–10933 (1994).

18. Aue, W. P., Bartholdi, E. & Ernst, R. R. *J. chem. Phys.* **64**, 2229–2246 (1976).
19. Griesinger, C., Otting, G., Wüthrich, K. & Ernst, R. R. *J. Am. chem. Soc.* **110**, 7870–7871 (1988).
20. Jeener, J., Meier, B. H., Bachmann, P. & Ernst, R. R. *J. chem. Phys.* **71**, 4546–4553 (1979).
21. Kay, L. E., Ikura, M., Tschudin, R. & Bax, A. *J. magn. Reson.* **89**, 496–514 (1990).
22. Fesik, S. W. & Zuiderweg, E. R. P. *J. magn. Reson.* **87**, 588–593 (1988).
23. Marion, D., Kay, L. E., Sparks, S. W., Torchia, D. A. & Bax, A. *J. Am. chem. Soc.* **111**, 1515–1517 (1989).
24. Otting, G. & Wüthrich, K. *J. magn. Reson.* **85**, 586–594 (1989).
25. Kraulis, P. J. *appl. Crystallogr.* **24**, 946–950 (1991).
26. Nicholls, A., Sharp, K. A. & Honig, B. *Proteins* **11**, 281–296 (1991).
27. Garner, L., Wills, J. W., Verderame, M. F. & Sudol, M. *Nature* **381**, 744–745 (1996).

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CORRESPONDENCE and requests for materials should be addressed to H.O. (e-mail: oschkinat@embl-heidelberg.de).

Crystal structure of a PDZ domain

João H. Morais Cabral*, Carlo Petosa*, Michael J. Sutcliffe†, Sami Raza†, Olwyn Byron*||, Florence Poy‡, Shirin M. Marfatia§, Athar H. Chishti§ & Robert C. Liddington*

* Departments of Biochemistry, || NCMH and † Chemistry, University of Leicester, Leicester LE1 7RH, UK

‡ Dana–Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, USA

§ Laboratory of Tumor Cell Biology, St Elizabeth's Medical Center, Tufts University School of Medicine, 736 Cambridge Street, Boston, Massachusetts 02135, USA

PDZ domains (also known as DHR domains or GLGF repeats) are ~90-residue repeats found in a number of proteins implicated in ion-channel and receptor clustering, and the linking of receptors to effector enzymes¹. PDZ domains are protein-recognition modules; some recognize proteins containing the consensus carboxy-terminal tripeptide motif S/TXV with high specificity^{2–4}. Other PDZ domains form homotypic dimers: the PDZ domain of the neuronal enzyme nitric oxide synthase binds to the PDZ domain of PSD-95, an interaction that has been implicated in its synaptic association⁵. Here we report the crystal structure of the third PDZ domain of the human homologue of the *Drosophila discs-large* tumour-suppressor gene product, DlgA. It consists of a

five-stranded antiparallel β -barrel flanked by three α -helices. A groove runs over the surface of the domain, ending in a conserved hydrophobic pocket and a buried arginine; we suggest that this is the binding site for the C-terminal peptide.

The PDZ domain⁶ is named after three of the proteins in which the repeats have been described: PSD-95 (postsynaptic density protein, M_r 95K), Dlg (*discs-large* protein) and ZO-1 (zonula occludens-1). These proteins have a conserved structure comprising three tandem PDZ domains, an SH3 domain and a guanylate-kinase-like domain. Other proteins that contain PDZ domains include certain protein kinases and protein tyrosine phosphatases, and neuronal nitric oxide (NO) synthase¹. These domains appear to be protein-recognition modules analogous to the well characterized SH2 and SH3 domains.

We crystallized a recombinant form of the third PDZ domain (PDZ-3) from human Dlg and solved its structure at 2.8 Å resolution using two heavy-atom derivatives and solvent flattening (Table 1). A complete model for the 96-residue domain has been built; in spite of a large proportion of glycine residues (13%), all of the secondary structure elements and connecting loops are well ordered. The domain is compact and globular, with a diameter of 25–30 Å. β -strands 2–5 form an up-and-down β -barrel and strand β 1 crosses over the barrel and hydrogen-bonds to β 5; a short α -helix (α 1) and its connecting loop cap one end of the barrel; helix α 2 caps the other end of the barrel, and a C-terminal helix (α 3) packs against the outside of the barrel (Figs. 1b and 2). Most of the conserved residues (Fig. 1a) are hydrophobic, and form the core of the domain, which is exposed on one face (Figs. 2, 3b). There are two exceptions: a conserved aspartic acid (D510), which is buried and forms a salt bridge to an arginine (R465); and an asparagine

(N516) in the $\beta 4$ - $\alpha 2$ loop whose side chain packs against the $\alpha 2$ - $\beta 5$ loop. Insertions and deletions in other members of the PDZ family are restricted to the connecting loops between secondary structure elements. One major insertion of six residues occurs in PDZ-1 and PDZ-2 of hDLG and PSD-95, which maps to the loop between strands $\beta 2$ and $\beta 3$. A well-ordered helix, $\alpha 3$, extends seven residues beyond the C terminus of the published consensus sequence for the PDZ family, and may well be present in all members of the family. If this is the case, no or very few residues link the C-terminal helix of PDZ domain 1 (PDZ-1) to the first strand of PDZ-2, consistent with the finding that a recombinant fragment containing both PDZ-1 and PDZ-2 forms a single protease-resistant module (S.M.M. and A.H.C., manuscript in preparation).

Several PDZ domains (the closely related second domains of Dlg (ref. 4) and PSD-95 (refs 2, 3) and one domain of PTP-BAS (ref. 7)) recognize peptides containing a consensus C-terminal T/SXV sequence (in single letter amino-acid code, where X is

any residue) with high specificity: mutation of the threonine to alanine or of the terminal valine to either alanine or aspartate abolishes binding². A large number of membrane-associated proteins, including many neuronal ion channels and synaptic receptors, share this terminal motif^{2,3}. We used the program SURFNET⁸ to search for peptide-binding cavities on the surface of the domain, which uses only geometric criteria derived from the atomic coordinates. In known protein-ligand complexes, the program correctly predicts the ligand-binding site (corresponding to the largest cavity) for 85% of the cases (J. Thornton, personal communication). For the PDZ domain, the program finds only one cavity (volume, 720 Å³) that is large enough for ligand binding (the volume of the second largest cavity is only 150 Å³). The cavity includes a hydrophobic pocket and a groove that runs over the top of the molecule (Fig. 3a). The hydrophobic pocket is highly conserved among PDZ family members (Fig. 3b) and is formed by the $\beta 1$ - $\beta 2$ loop (which includes the conserved GLGF motif) and side chains from strand $\beta 2$ and helix $\alpha 2$ (Figs 2, 3c). At the

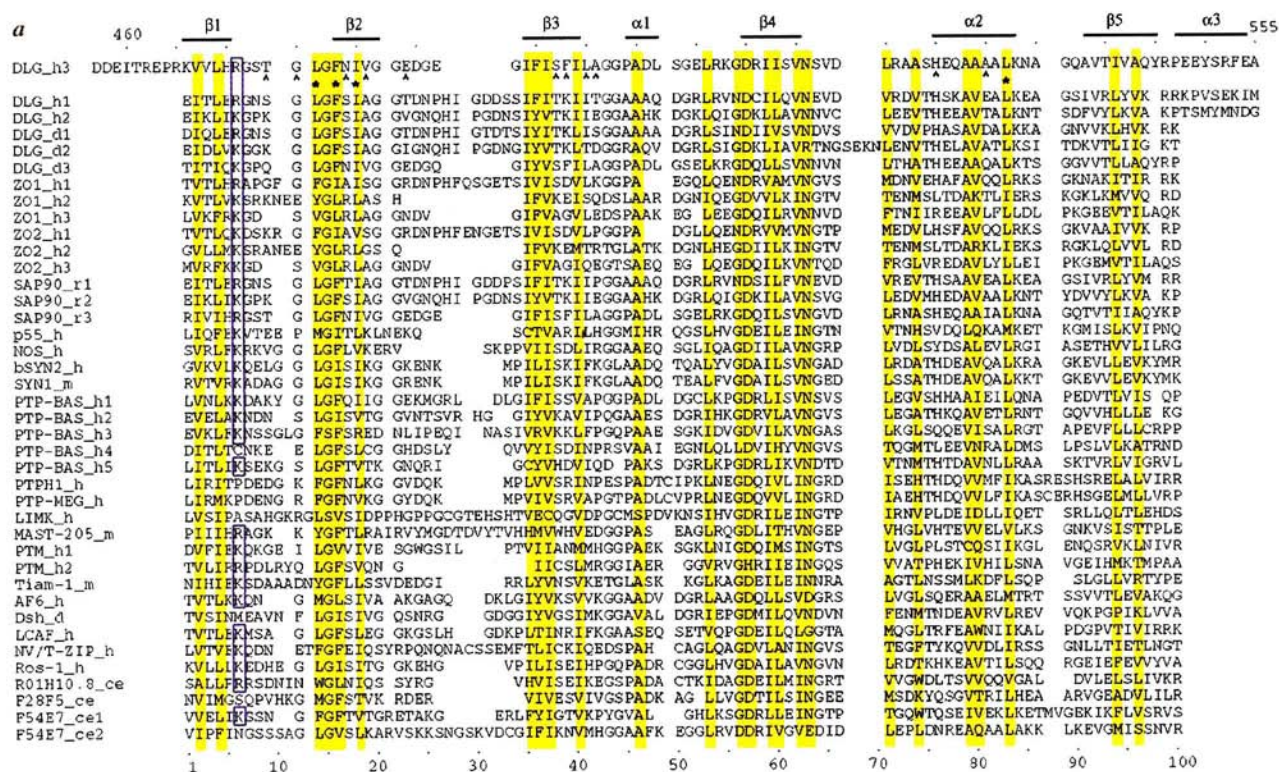
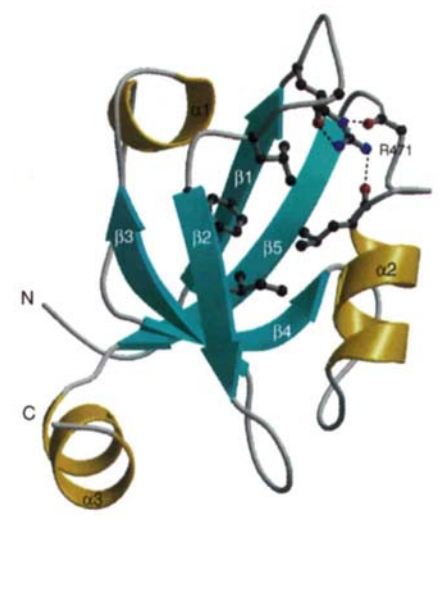


FIG. 1 a, Sequence alignment of the PDZ domain family, with secondary structure indicated for the PDZ-3 domain of hDlg (DLG_h3). Residue numbering at the top is that of the full-length hDlg protein. Positions where the chemical character of residues is conserved in 90% of sequences are highlighted in yellow. The four residues forming the hydrophobic pocket are marked by asterisks. The sequences of domains PDZ-1 and PDZ-2 from hDlg are contiguous. The sequences are from various species (h, human; r, rat; m, mouse; d, *Drosophila*; ce, *C. elegans*). The alignment and the sequence numbering shown at the bottom were also taken from ref. 1, where a full explanation of the sequence abbreviations can be found. The largely conserved basic residue corresponding to R471 in hDlg is boxed. The principal residues involved in dimerization are T474, G475, N479, V481, E484, S492, F493, L495, A496, H525 and A530 and are indicated with a caret (^). b, Stereo C α plot of PDZ-3, with every tenth residue numbered and the N and C termini indicated. The fold-

search program DALI¹⁹ specifies the β -subunit of *Klebsiella aerogenes* urease²⁰ as having a similar fold, classified by the SCOP database²¹ as a 'β-clip' fold, which is also shared by the enzyme dUTPase²².



◀ FIG. 2 Ribbon diagram of the PDZ-3 domain with secondary structure elements and N and C termini indicated, generated with MOLSCRIPT²³, RASTER3D²⁴ and RENDER²⁵. Side chains forming the hydrophobic pocket (residues Leu 476, Phe 478, Ile 480 and Leu 532), along with Arg 471, are also shown.

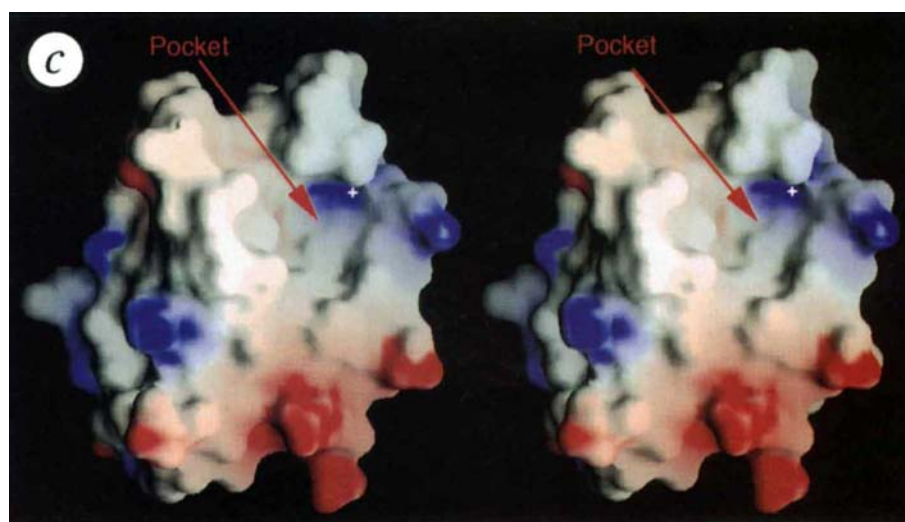
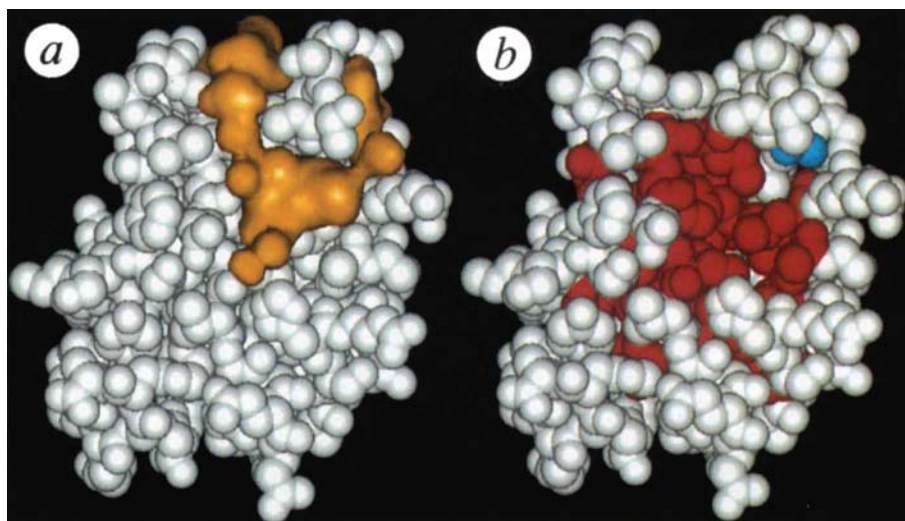


FIG. 3 Surface of the PDZ-3 domain. The view shown is similar to that in Fig. 2. *a*, Space-filling model with the largest cavity found by SURFNET⁸, shown in gold. *b*, Space-filling model showing conserved residues. Residues in red are those that are highly conserved among PDZ domains. The side-chain nitrogens of Arg471 are shown in blue. *c*, Stereo surface-charge representation, generated with the program GRASP²⁶. Regions with positive and negative electrostatic potential are shown in blue and red, respectively. *d*, Close-up of *c*, with the model tripeptide TDV shown in the hydrophobic pocket.

METHODS. A selection of probes (-OH, CO₂, -CO, -CH₃, -NH) was used to search the SURFNET⁸ cavity for possible binding sites using GRID²⁷. The tripeptide was then manually docked into the cavity using the contour maps from GRID as a guide. Weak harmonic restraints were then applied to enable the automatic generation by molecular dynamics and simulated annealing (MODELLER²⁸) of a set of 10 three-dimensional models of the tripeptide complexed with the PDZ domain. The model with the lowest energy was selected.

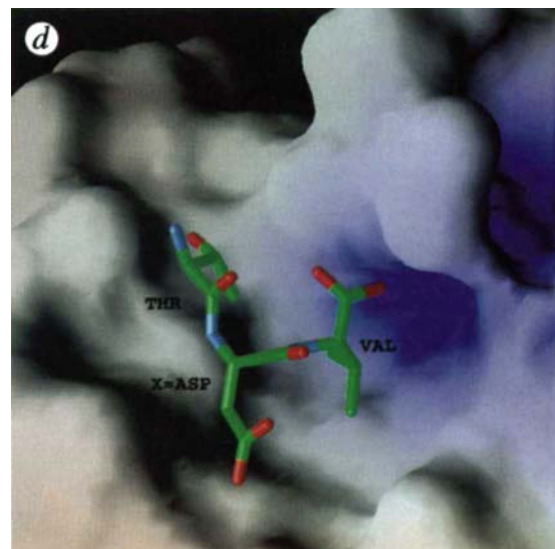


TABLE 1 Summary of crystallographic analysis

Crystals	Native	K ₂ PtCl ₄	CH ₃ HgNO ₃		
Resolution (Å)	2.8	3.5	5.0		
Unique reflections	5,793	1,998	1,014		
Completeness (%)	100	80.1	98.6		
R _{merge} (%) (outer shell)	10.8 (30.1)	8.6 (12.6)	4.0 (4.4)		
Redundancy	13	2.5	9		
R _{iso} (%)		9.6	5.6		
No. of sites		3	2		
Phasing power		1.5	0.62		
Figure of merit, 0.34 (after DM ¹⁴ 0.76)					
Refinement statistics					
Resolution	No. of atoms	R _{cryst} (%)	R _{free} (%)	R.m.s. bond lengths (Å)	R.m.s. bond angle (deg)
15–2.8 Å	727	22.0	27.1	0.008	1.4

$R_{\text{merge}} = \sum_i |I_i - \langle I \rangle| / \sum_i I_i$, where I_i is the observed intensity and $\langle I \rangle$ is the average intensity. $R_{\text{so}} = \sum_i |F_{\text{ph}} - F_p| / \sum_i |F_{\text{ph}}|$, where F_{ph} is the heavy-atom derivative structure factor and F_p is the protein structure factor. Phasing power is $\langle |F_{\text{ph}}| \rangle / \langle E \rangle$, where E is the residual lack of closure error. Figure of merit is $\langle |\Sigma P(\alpha) e^{i\alpha} / \Sigma P(\alpha)| \rangle$, where $P(\alpha)$ is the phase probability distribution. $R_{\text{cryst}} = \sum_i |F_o - F_{\text{p(calc)}}| / \sum_i F_o$ for reflections with $F_o > 2\sigma F_o$. R_{free} is the same as R_{cryst} , but calculated on the 10% of data excluded from refinement. The domain was expressed as a glutathione-S-transferase (GST) fusion in *E. coli* strain BL21. The expressed protein includes residues 457 to 552 of the human homologue of *Drosophila discs-large* protein¹¹, and was purified on glutathione-Sepharose (Pharmacia) and eluted after protease digestion with thrombin. It was further purified on Mono-Q Sepharose (Pharmacia). The crystals grow by hanging-drop vapour diffusion at a protein concentration of 15 mg ml⁻¹ from 0.9–1.3 M sodium citrate, pH 6.5–7.5 at 4 °C, and adopt space group P6₃22, with $a = 111.0$ Å, $c = 62.5$ Å. All diffraction data were collected with a Rigaku RU200HB X-ray generator and image plate with Cu-K α radiation. Data were processed using DENZO and SCALEPACK¹². One platinum position was found from the difference Patterson map with the program RSPS¹³, and further sites were derived from difference Fourier after solvent flattening and histogram matching using the program DM¹⁴ from the CCP4 program suite¹⁵. Heavy-atom parameters were refined using HEAVY¹⁶ and MLPHARE¹⁵. The high solvent content (70%) present in the crystal was a powerful constraint during phase refinement and extension and allowed the production of an easily interpretable electron-density map¹⁷. The refinement consisted of rounds of simulated annealing and grouped B-factor refinement to 2.8 Å with XPLOR¹⁸. The present model includes 96 residues, from residues 460 to 552, plus three residues at the C terminus from the expression vector, and eight water molecules. All main-chain torsion angles, except S517, in the $\beta 4$ – $\alpha 2$ loop, fall in allowed regions of the Ramachandran plot. The relative molecular mass and K_d were determined by sedimentation equilibrium in a Beckman Optima XL-A analytical ultracentrifuge using a Marquardt–Levenberg non-linear least-squares-fitting model, IDEAL1, and MULTI/SELF, a modified Gauss–Newton, four-exponent, self-association model included in the Beckman data analysis software. The rotor speed was 30,000 r.p.m. and equilibrium solute distributions, achieved within 18 h at 20 °C, were recorded for seven solute concentrations in the range 0.02–1.0 mg ml⁻¹, with scanning ultraviolet optics at wavelengths of 220 and 278 nm.

back of the pocket is a partially buried arginine, R471, from the $\beta 1$ – $\beta 2$ loop, which is held in a rigid conformation by hydrogen bonds to three main-chain carbonyl oxygens, and is reminiscent of the buried arginine involved in phosphotyrosine binding to SH2 domains⁹. A positively charged residue (arginine or lysine) is present in this position in all PDZ domains that are known to bind C-terminal peptides (and in ~85% of known PDZ domains). We modelled a consensus tripeptide, TDV, into the putative binding site, to see if the peptide could be accommodated into this cavity with good stereochemistry and complementarity of non-covalent interactions (Fig. 3d). The lowest-energy model meets these criteria, and orients the valine side chain into the hydrophobic pocket, with the terminal carboxylate salt-bridging to the arginine. The aspartic acid side chain points out into solution; the threonine hydroxyl makes a hydrogen bond with the carbonyl group of a conserved glycine (G477). The shortest peptides that bind PDZ domains are nine residues long; residues upstream of the C-terminal tripeptide could be accommodated within the groove that extends over the top of the domain. The modelling

therefore shows that the cavity is a good candidate for the binding site, but crystal structures of PDZ–peptide complexes are required to define the details of the protein–peptide interactions and the molecular basis of specificity.

Homotypic dimer formation between PDZ domains plays a role in the localization of nNOS⁵, and it has been suggested that self-association of PSD-95 proteins is necessary for receptor clustering¹⁰. In the crystal, the domain forms a dimer about a crystallographic two-fold axis, burying 13% of the monomer surface (total buried surface, 1,440 Å²). A ridge formed by the exposed residues in strands $\beta 2$ and $\beta 3$ contacts the surface surrounding the putative peptide-binding pocket in a second molecule. To investigate a possible functional role for the PDZ-3 dimer, we measured its dissociation constant in solution by analytical ultracentrifugation (Table 1). The value obtained, $K_d = 2 \pm 1$ mM, shows that the dimer interaction is weak. However, at sites of receptor and ion-channel clustering where the local concentration of PDZ-containing proteins is very high, it is possible that self-association through this domain is important physiologically. □

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- Ponting, C. P. & Phillips, C. *Trends Biochem. Sci.* **20**, 102–103 (1995).
- Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N. & Sheng, M. *Nature* **378**, 85–88 (1995).
- Kornau, H. C., Schenker, L. T., Kennedy, M. B. & Seeburg, P. H. *Science* **269**, 1737–1740 (1995).
- Matsumine, A. et al. *Science* **272**, 1020–1023 (1996).
- Brennan, J. E. et al. *Cell* **84**, 757–767 (1996).
- Kennedy, M. B. *Trends Biochem. Sci.* **20**, 350 (1995).
- Sato, T., Irie, S., Kitada, S. & Reed, J. C. *Science* **268**, 411–415 (1995).
- Laskowski, R. A. *J. Mol. Graph.* **13**, 323–330 (1995).
- Waksman, G. et al. *Nature* **358**, 646–653 (1992).
- Gomperts, S. N. *Cell* **84**, 659–662 (1996).
- Lue, R. A., Marfatia, S. M., Branton, D. & Chishti, A. H. *Proc. Natl Acad. Sci. USA* **91**, 9818–9822 (1994).
- Otwiński, Z. & Minor, W. in *Data Collection and Processing* (eds Sawyer, L., Isaacs, N. & Bailey, S.) 556–562 (SERC Daresbury Laboratory, Warrington, 1993).
- Knight, S. thesis, Swedish Univ. Agric. Sci. Upsalla (1989).
- Cowan, K. *Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography* **31**, 34–38 (1994).
- CCP4 Acta Crystallogr. **D50**, 760–763 (1994).
- Terwilliger, T. & Eisenberg, D. *Acta Crystallogr.* **A39**, 813–817 (1983).
- Jones, T. A. *Methods Enzymol.* **115**, 157–171 (1985).

- Brünger, A. *XPLOR Version 3.1: A system for X-Ray Crystallography and NMR* (Yale University Press, New Haven, Connecticut, 1992).
- Holm, L. & Sander, C. *J. Mol. Biol.* **233**, 123–138 (1993).
- Jabri, E., Carr, M. B., Hausinger, R. P. & Karplus, P. A. *Science* **268**, 998–1004 (1995).
- Murzin, A. G., Brenner, S. E., Hubbard, T. & Chothia, C. *J. Mol. Biol.* **247**, 536–40 (1995).
- Cedergren-Zeppezauer, E., Larsson, G., Nyman, P. O., Dauter, Z. & Wilson, K. *Nature* **355**, 740–743 (1992).
- Kraulis, P. J. *J. Appl. Crystallogr.* **24**, 946–950 (1991).
- Bacon, D. J. & Anderson, W. F. *J. Mol. Graph.* **6**, 219–220 (1988).
- Merritt, E. A. & Murphy, M. E. P. *Acta Crystallogr.* **D50**, 869–873 (1994).
- Nicholls, A., Sharp, K. A. & Honig, B. *Proteins Struct. Funct. Genet.* **11**, 281–296 (1991).
- Goodford, P. J. *J. Med. Chem.* **28**, 849–857 (1985).
- Sali, A. & Blundell, T. L. *J. Mol. Biol.* **234**, 779–815 (1993).

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CORRESPONDENCE and requests for materials should be addressed to R.C.L. (e-mail: rcl6@leicester.ac.uk).

Chemical characterization

Making a splash this week — halogen heater moisture analysers, a new sequential ICP atomic emission spectrometer and a chemical reaction search system.

CEM, a developer of microwave technology for laboratory applications, has introduced its simultaneous temperature-accelerated reactions (STAR) **digestion system**. The system is designed for large sample sizes and/or hard-to-digest samples, and brings microwave speed to open-cavity digestions, producing samples on demand. Special features include independent position vessels that let each vessel (up to six) run a separate digestion method, staggered start times to allow the system's individual positions to fit into the laboratory workflow, optional program-controlled reagent addition during digestion and individual temperature feedback control for highly reproducible results.

Reader Enquiry No. 100

The IKA-Calorimeter C 5000 was created for the **determination of combustion and caloric values in liquid and solid samples** with simultaneous halogen and sample decomposition. Optional methods offered by this system are adiabatic, isoperibolic, isothermal and semi-dry. In addition, the measurable parameters after sample decomposition include combustion value, caloric value, as well as fluorine, chlorine and sulphur concentration. System features include the automatic filling of external



The automated IKA-Calorimeter C 5000.

decomposition vessels with oxygen, a dry cooling system, reduction of measurement time and features that help to conform with international standards in design and function (DIN 51900, ISO 1928, ASTM 240 d, BSI). The systems are designed to be efficient and maintenance-free, using an internal water handling system that is completely independent of the water supply system. The C 5000 offers several advantages when compared with conventional calorimetric evaluation, including a high degree of automation with all process currents controlled by a central control com-

puter and a choice of measurement methods. For overview measurements, a reduction of the measurement time can be utilized (systems with a reduction of the measurement time in standard operations neither conform to DIN nor to any other standard).

Reader Enquiry No. 101

A new range of **laboratory furnaces** for burn-off, ashing, loss-on-ignition and calcining procedures at temperatures up to 1,200 °C has been introduced by Carbolite. The BWF furnaces have been designed for burn-off cleaning processes requiring a large airflow. Elements that are graded to compensate for heat loss and provide good temperature uniformity are housed in the sides of the chamber to prevent contamination by accidental spillage. The furnaces have a 13-litre capacity with 1,100 °C and 1,200 °C maximum temperatures. OAF furnaces, on the other hand, are designed for ashing a wide variety of materials from food to plastics at temperatures up to 1,100 °C, and have low chambers to hold hot air close to the samples. This feature, together with a generous airflow, results in rapid burning. Large floor areas allow many samples to be accommodated at one time. Single- and double-chamber versions are available. For analytical techniques that are sensitive to alumina or silica dust, the company offers the GSM furnace. This furnace incorporates a fused quartz muffle, which minimizes the risk of dust falling on to the samples. This design also keeps corrosive vapours away from the heating elements. The GSM model operates up to a temperature of 1,100 °C.

Reader Enquiry No. 102

A new type of **moisture analyser**, using halogen heater technology, has been launched by Mettler-Toledo. The halogen heating technology is said to be reliable and controllable, giving accurate and reproducible results. Samples can be heated up to 200 °C within 30 s with a minimum of sample decomposition, according to the manufacturer. The analyser is the company's first to use prompting symbols, with a back-lit screen to keep the user continuously updated with details concerning the operating status. A CD-style drawer makes sample loading easy, and the sealed keypad is constructed to be tough enough to withstand constant use in less than ideal conditions. A databank can permanently



Mettler-Toledo's moisture analyser.

store methods for up to 20 substances, with a standard data interface allowing information to be taken directly from the instrument to a PC for analysis. Both weight and temperature can be calibrated and recorded, using a clip-on printer, helping to comply with good laboratory practice. Two models are available: the HG53, which is the standard model for routine work in laboratories, production areas or for quality control, and the HR73, which has an extra 20-g weight range, more flexible temperature step setting, and manageable record memory and statistics. The halogen heating method should prove useful for thermogravimetric analysis.

Reader Enquiry No. 103

Ocean Optics has developed a line of **spectrometer-coupled fibre-optic sensors** that monitor colour changes in immobilized indicator dyes to measure pH or toxic metals. The systems consist of a single or dual PC-card-mounted spectrometer, including a tungsten-halogen light source, transmission or reflection film probe, as well as a selection of reactive polymer films. A Windows-based pH software package, pHiber Optical, collects data from the sensors and 4–20 mA inputs from a conductivity/temperature meter to monitor and display pH. Also available from the company is Ocean32, a version of Galactic Industries' Grams/32. It includes drivers to acquire spectra directly from Ocean Optics' spectrometers, operates on IBM-compatible 386 (or better) PCs with a maths coprocessor and a recommended minimum of 8 MB of RAM. The program should prove useful for users who require data acquisition and processing, advanced graphics, spectral desktop publishing, and a comprehensive set of analytical and mathematical tools.

Reader Enquiry No. 104

Packard Instruments' Radiomatic **radio-HPLC detectors**, with significant signal-to-noise improvements, can detect low-level sample metabolites, impurities and other radiolabelled peaks with sensitivities that are stated to be beyond the capabilities of conventional radio-HPLC detectors. According to the manufacturer, this system can increase sensitivities by up to 300 per cent using a 1,024 multichannel analyser and Packard's time-resolved liquid scintillation counting technology (TR-LSC). Real-time analysis of the radio-isotopic spectrum, with automatic spectral region optimization, eliminates interferences from background and luminescence.

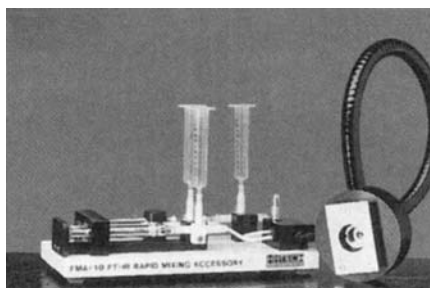
Reader Enquiry No. 105

FTIR analysis

ATI Unicam's **Infinity series of FTIR** is designed to change sources, beamsplitters and detectors automatically, allowing coverage of the visible, near-, mid-, far-IR spectral ranges as part of an automated method. The computer-controlled beamsplitter carriage is housed within a sealed and desiccated chamber. Previously, manual changing of the beamsplitter carried a risk of damaging this expensive equipment. This could also delay operation by as much as 30 min while the purge is re-established, the temperature is re-equilibrated and the system is retuned. Now, components move in or out of the system, and previous settings for each spectral range are set up automatically, eliminating the need to revalidate the system. Within this series of instruments, the analyst is able to choose from interferometer/resolution, sources, beamsplitters, detectors, external beams, auxiliary sample compartments, as well as a number of 'hyphenated techniques'. Method development is said to be simplified, automatically surveying the entire spectral range then optimizing the source/beamsplitter/detector configuration. Users can automate the entire method through WinFirst's ApPro macros, Builder and Wizards, before transferring the method to a dedicated QC-type spectrometer, such as Genesis.

Reader Enquiry No. 106

The **FMA-10 rapid mixing accessory**, developed by Hi-Tech Scientific, allows FTIR spectroscopy to be used in stopped-flow kinetic studies. This accessory brings an additional detection method to a technique more normally associated with fluorescence and absorbance spectrophotometric methods. It features a sample cell that has been designed with a highly efficient mixing system. The optical windows are manufactured from zinc selenide or calcium fluoride (by request) for good IR transmission. Rapid injection of the reagents allows reactions with half lives as low as 20 ms to be followed. A thermo-



The FMA-10 rapid FTIR mixing accessory.

stat-controlled flow circuit allows precise temperature control over the 5–80 °C range. This accessory is compatible with most FTIR spectrometers using a 3 × 2 inch mount. The unit utilizes standard stopped-flow principles with a stopping syringe, and the range of accessible reactions is further extended by the provision of a continuous flow mode.

Reader Enquiry No. 107

Atomic absorption

Varian has introduced a new **sequential ICP atomic emission spectrometer** for the analysis of difficult samples, which includes multitasking software based on the Windows95 operating system. The Liberty series II is said to analyse difficult organic solvents, such as methanol, without dilution, and to improve detection limits by a stated 50 per cent over its predecessor. Varian's new Plasma96 software employs an easy-to-use worksheet concept modelled on an analyst's workbook. From the central worksheet, users can rapidly access all results and graphics during the fully automated analysis. The worksheet can also be used to customize reports quickly from stored data or select subsets of elements for analysis. Available as an upgrade to Liberty ICP users, Plasma96 permits 'urgent' samples to be inserted into an automatic sequence for rapid analysis, and includes quality-control, data-reporting and export options.

Reader Enquiry No. 108

With the introduction of the AAnalyst 100 and AAnalyst 300 **atomic absorption spectrometers**, Perkin-Elmer has combined cost-effectiveness and ease of use with improved analytical power. These spectrometers share many features, including automated wavelength and slit selection, the optical system, a large sample compartment, a six-lamp motorized turret (optional on the AAnalyst 100 spectrometer) and a newly designed burner system. The six-lamp turret features automatic lamp alignment, further simplifying the analyst's job. Both systems use the new Lumina cableless hollow-cathode lamps and the new GemTip nebulizer. The model 300 system is fully automated, providing sequential multiple element capabilities with a built-in deuterium back-

ground corrector and a computer-controlled gasbox. All functions are controlled by a stand-alone PC using AA WinLab software.

Reader Enquiry No. 109

Reaction information systems

Cognos from InfoChem, a new reaction information system for the Macintosh, provides chemists with **fast and easy access to large chemical reaction databases**. This chemical reaction search system is based on a new concept for reaction indexing developed by researchers at Brandeis University. The system includes a database of over 390,000 chemical reactions and is capable of operating on multiple databases in real time, permitting post-search modification of the query while continuously showing the number of retrievals as modifications are effected. Cognos is supplied complete with the ChemReact database and provides fast and easy access to 390,000+ reaction types. InfoChem has derived ChemReact from a larger database of 2.5 million reactions cited in the literature between 1975 and 1991.

Reader Enquiry No. 110

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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